PCT

526 Rec'd PCT/PTO 19 OCT 2000

FORM PTO-1390 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE
(REV 5-93)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 030307/0191

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): Unassigned 09/673617

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DK99/00209	April 14, 1999	April 21, 1998

TITLE OF INVENTION
FOOD-GRADE CLONING VECTOR AND THEIR USE IN LACTIC ACID BACTERIA

APPLICANT(S) FOR DO/EO/US
KIM SORENSEN, Rasmus LARSEN, Eric JOHANSEN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - ☒ has been transmitted by the International Bureau.
   - ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ have been transmitted by the International Bureau.
   - ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A copy of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Unassigned 09/673617	PCT/DK99/00209	030307/0191

17. ☒ The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO $860.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2) $710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO $1,000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))		

Claims	Number Filed		Included in Basic Fee		Extra Claims		Rate	
Total Claims	40	-	20	=	20	×	$18.00	$360.00
Independent Claims	1	-	3	=	0	×	$80.00	$0.00
Multiple dependent claim(s) (if applicable)							$270.00	
TOTAL OF ABOVE CALCULATIONS =								$1,220.00
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).								$0.00
SUBTOTAL =								$1,220.00
Processing fee of $130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f). +								
TOTAL NATIONAL FEE =								$1,220.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +								
TOTAL FEES ENCLOSED =								$1,220.00
							Amount to be: refunded	$
							charged	$

a. ☒ A check in the amount of $1,220.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 19-0741 in the amount of $0.00 to the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0741. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGISTRATION NUMBER 29,768

Atty. Dkt. No. 030307/0191

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: KIM SORENSEN et al.

Title: FOOD-GRADE CLONING VECTOR AND THEIR USE IN LACTIC ACID BACTERIA

Appl. No.: Unassigned

Filing Date: October 19, 2000

Examiner: Unassigned

Art Unit: Unassigned

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, Applicants respectfully request that the above-identified application be amended as follows:

In the Claims:

Please amend the following claims:

Claim 15 line 1, delete "any of claims 1-14" and insert

--claim 1--.

Claim 22 line 1, delete "any of claims 1-21" and insert

--claim 1--.

Claim 32, lines 1 and 2, delete "any of claims 22-31" and insert

--claim 22--.

Atty. Dkt. No. 030307/0191

REMARKS

Applicants respectfully request that the foregoing amendments to Claims 15, 22 and 32 be entered in order to avoid this application incurring a surcharge for the presence of one or more multiple dependent claims.

Respectfully submitted,

Date October 19, 2000

By ______________________

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FOOD-GRADE CLONING VECTOR AND THEIR USE IN LACTIC ACID BACTERIA

FIELD OF INVENTION

The present invention relates to the field of lactic acid bacterial starter cultures and in particular there is provided a food-grade vector comprising a nonsense mutation suppressor-encoding gene which vector, when it is present in a lactic acid bacterial strain, permits such a strain to have an industrially appropriate growth rate and metabolic activity.

TECHNICAL BACKGROUND AND PRIOR ART

Lactic acid bacteria are used extensively as starter cultures in the food industry in the manufacturing of fermented products including milk products such as e.g. yoghurt and cheese, meat products, bakery products, wine and vegetable products. *Lactococcus* species including *Lactococcus lactis* are among the most commonly used lactic acid bacteria in dairy starter cultures. However, several other lactic acid bacteria such as *Leuconostoc* species, *Pediococcus* species, *Lactobacillus* species and *Streptococcus* species are also commonly used in food starter cultures.

A significant role of lactic acid bacteria is to render the fermented products microbiologically stable and to improve the taste and palatability of these products. It is generally recognised that genes, the expression of which are important to ensure that the addition of lactic acid bacteria to a starting material results in the desired fermentation effect, are found naturally or can be inserted on extrachromosomal DNA vectors including plasmids.

However, DNA vectors may be unstable, resulting in their loss from the cells. Accordingly, it is of pertinent industrial interest to provide vectors which are stably maintained in lactic acid bacterial starter cultures.

Presently used methods of stably maintaining (stabilising) vectors in a host cell include insertion of relatively large DNA sequences such e.g. antibiotic or bacteriocin resistance

genes into the cell. In the art, such genes are also referred to as selection markers. However, it is well-known that the insertion of large DNA sequences involves the risk that other sequences are deleted from the vector. Furthermore, the use of resistance genes for maintaining the plasmid in the host cell implies that antibiotics or bacteriocins must be present in the cultivation medium. This is undesirable in the manufacturing of food and feed products. In addition, it is undesirable that live bacteria comprising antibiotic resistance genes are present in food products as such genes may be transferable to the indigenous gastro-intestinal microflora.

Consequently, there have been reported several attempts to develop so-called food-grade cloning vectors. In the present context, the term "food-grade" indicates that the vector consists essentially of DNA of lactic acid bacterial origin.

WO 91/09131 discloses a vector essentially consisting of lactic acid bacterial DNA wherein a gene coding for the bacteriocin nisin is used as a selectable marker. However, the selection of such a vector still requires that a selective compound is added to the cultivation medium.

As an alternative approach, it has been suggested to use vectors carrying a gene coding for a gene product that suppresses nonsense mutations in lactic acid bacteria.

In the *in vivo* synthesis of proteins occurring in the ribosomes, mRNA is translated into polypeptide chains. However, the mRNA codons do not directly recognise the amino acids that they specify in the way that an enzyme recognises a substrate. Translation uses "adaptor" molecules that recognise both an amino acid and a triplet group of nucleotide bases (a codon). These adaptors consist of a set of small RNA molecules known as transfer RNAs (or tRNAs), each of which is only 70 to 90 nucleotides in length. Such tRNA molecules contain unpaired nucleotide residues comprising a CCA triplet at one end of the molecule and, in a central loop, a triplet of varying sequence forming the so-called anticodon that can base-pair to a complementary triplet in the mRNA molecule, while the CCA triplet at the free 3' end of the molecule is attached covalently to a specific amino acid.

The three nucleotide triplets UAG (amber codon), UGA (opal codon) and UAA (ochre codon) do not code for an amino acid. These signals termed stop codons or "nonsense"

codons, are involved in polypeptide chain termination. During translation, two protein factors (R1 and R2) recognise these triplets and effect release of the polypeptide chain from the ribosome-mRNA-tRNA complex.

Occasionally a mutation occurs in a cell resulting in a nonsense codon appearing within a gene, causing premature chain termination and the production of a protein fragment. Such fragments rarely have enzymatic activity.

The effect of such a nonsense mutation can be reversed or suppressed by a second mutation in a gene coding for a tRNA which results in the synthesis of an altered tRNA molecule. Such an altered tRNA recognises a nonsense codon and inserts an amino acid at that point in the polypeptide chain. The mutated tRNA-encoding gene is termed a suppressor gene and the altered nonsense mutation-suppressing tRNA which it encodes is generally referred to as a nonsense or termination suppressor. Such termination suppressors may be derived by single, double or triple base substitutions in the anticodon region of the tRNA.

Most mutations in a tRNA-encoding gene leading to the formation of a nonsense suppressor are located in the anticodon triplet and alter it to CUA, UUA or UCA. Such suppressors may be referred to as amber, ochre and opal suppressors, respectively. Following the rules of nomenclature of Demerec et al., 1966 which was suggested for termination (nonsense) suppressors in *E. coli* the symbol "*sup*" and assigned capital letters as gene designations, e.g. *supB, supC* or *supZ*, are used herein also to designate suppressor genes in lactic acid bacteria.

In Dickely et al. 1995, Johansen et al. 1995 and WO 95/10621 are disclosed plasmids containing a gene coding for a tRNA that is a suppressor for a nonsense mutation where the suppressor gene will function as a selectable marker when the nonsense mutation in the host strain for the plasmid is one which, in the absence of a corresponding suppressor gene, will render the host strain incapable of growing in a particular environments, such as e.g. milk or other food or feed products. The genes coding for suppressor tRNA are small and can be inserted without causing deletions of desired genes. Also, homologous recombination will not occur between *supD* and the chromosomal tRNA genes due to the small size.

The construction of the vector pAK89.1 that comprises a *supD* suppressor is described in Dickely et al. 1995 and WO 95/10621. However, this cloning vector contains a gene coding for erythromycin resistance and thus is not a food-grade vector.

Dickely et al. 1995 and WO 95/10621 disclose food-grade vectors based on the *Lactococcus lactis* derived nonsense suppressor, *supB*, as a selection marker. However, these vectors, pFG1 and pFG1.1, cause growth inhibition when present in host cells. It has also been found that these particular vectors, when present in lactic acid bacterial strains, are unstable and that the acidification rate of the host cells in milk is reduced as compared to wildtype strains and therefore these vectors are not suitable in industrial processes.

Accordingly, the prior art is not aware of nonsense suppressor containing food-grade vectors that are stably maintained in lactic acid bacterial strains and which do not adversely affect the growth and metabolic activity of the host strains.

The present invention provides a food-grade vector, comprising as the selection marker a nonsense suppressor gene. It was surprisingly found, that the vector, when it is present in a lactic acid bacterial strain comprising a nonsense mutation suppressible by the suppressor, substantially does not cause growth inhibition and permits the strain to acidify milk at essentially the same rate as that of the same strain not containing the vector.

SUMMARY OF THE INVENTION

Accordingly, the present invention relates in a first aspect to a recombinant vector consisting essentially of lactic acid bacterial DNA, the vector comprising a gene coding for a tRNA comprising an amber suppressor and a replicon making the vector capable of replicating in a lactic acid bacterium, the vector having at least one of the following characteristics:

(i) when it is present in *Lactococcus lactis* strain FA4-1-1 deposited under the accession No. DSM 12086 having an amber mutation in the *pyrF* gene that is suppressible by the suppressor, it permits said strain to grow at 30°C at a doubling time of at the most 100 minutes in a minimal medium not containing pyrimidine sources; and/or

(ii) when it is present in a strain of *Lactococcus lactis* FH CY-1 that has an amber mutation in the *pyrF* gene (strain CHCC4146, DSM 12109), said amber mutation being suppressible by the suppressor, it permits the strain to acidify milk under identical conditions at essentially the same rate of that of the parent strain (FH CY-1) deposited under the accession No. DSM 12087;

and/or (iii) it permits the *Lactococcus lactis* FA4-1-1 strain (DSM 12086) to grow at 30°C in a minimal medium not containing pyrimidine sources at a doubling time which is less than that for the *Lactococcus lactis* strain DN209 transformed with the vector pFG1.1 deposited under the accession No. DSM 12088, the pFG1.1 vector comprising a gene coding for a suppressor that is capable of suppressing the nonsense mutation in the DN209 strain, the transformed DN209 strain growing under conditions identical to those for the FA4-1-1 strain.

The present invention also pertains to a lactic acid bacterium comprising a vector according to the invention, the lactic acid bacterium possibly comprising an amber mutation being suppressible by the nonsense amber suppressor.

In a further aspect, the invention relates to an isolated pure culture of a lactic acid bacterium according to the invention, to a composition comprising such an isolated pure culture, and a carrier, and to the use of such a composition as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

In one interesting aspect, the invention relates to a method of stably maintaining a vector according to the invention in lactic acid bacterial host cells growing in a particular environment, comprising providing said host cells as nonsense mutant cells having lost the capability of growing in said environment, and transformed with a plasmid according to the invention containing a nonsense suppressor gene encoding a gene product restoring the capability of the nonsense mutant cells to grow in said environment whereby, if the plasmid is lost from the lactic acid bacterial cells, the cells will not grow.

DETAILED DISCLOSURE OF THE INVENTION

Thus, it is an important objective of the present invention to provide a recombinant vector consisting essentially of lactic acid bacterial DNA, the vector comprising a gene coding for a tRNA comprising an amber suppressor and a replicon making the vector capable of replicating in a lactic acid bacterium and having at least one of the characteristics (i) to (iii) as mentioned above.

As used herein, the term "vector" is used interchangeable with the terms "recombinant vector", "cloning vector" and "expression vector", and relates to any DNA molecule that acts as an intermediate carrier into which a gene or a DNA segment is inserted for introduction into bacterial or other cells for amplification. Such intermediate carriers include DNA fragments or subsequences, plasmids, cosmids, bacteriophages and transposons.

When used herein, the term "lactic acid bacterium" designates a group of bacteria having as a common characteristic the capability to produce lactic acid from sugars. The majority of the species belonging to this group can be characterised as gram-positive, catalase-negative, microaerophilic or anaerobic bacteria which may be cocci or rods. The anaerobic genus *Bifidobacterium* is also generally included in the group of lactic acid bacteria.

The recombinant vector according to the invention consists essentially of DNA of lactic acid bacterial origin including DNA isolated from vectors or other replicons having the lactic acid bacterium as their natural host organism. In the art, such vectors are, as it is mentioned above, also referred to as being "food grade" vectors, since it is generally considered that the use of such vectors may be allowable by relevant governmental authorities for use in food manufacturing.

In the present context, the expression "amber mutation" relates to a mutation in a cell resulting in the nonsense codon UAG appearing within a coding sequence of a gene resulting in premature chain termination. The effect of such amber mutations can be reversed or suppressed by an "amber suppressor" i.e. a tRNA comprising an altered anticodon, CUA, which only recognises amber mutations and which is the result of at least one change of nucleotide in a gene coding for a tRNA anticodon (Eggertson et al., 1988).

As mentioned above, one characteristic of the vector of the present invention is that it, when it is present in *Lactococcus lactis* strain FA4-1-1 (DSM 12086) having an amber mutation in the *pyrF* gene that is suppressible by the amber suppressor, permits said strain to grow at 30°C at a doubling time of at the most 100 minutes in a medium not containing pyrimidine sources. In preferred embodiments the vector permits the above strain to grow at a doubling time of at the most 95 minutes such as at the most 90 minutes including at the most 85 minutes.

It will be understood, that an amber mutation in a *pyr* gene of a lactic acid bacterial cell causes the cell to lose its capability to grow in a medium, like e.g. milk, which does not contain pyrimidines. Such an auxothrophic mutant will only be able to grow in the absence of pyrimidine precursor if the vector of the present invention is present in the host cell. Thus, the amber suppressor restores the capability of the cell to grow in such a medium.

As it is also mentioned above, it is of industrial interest that a vector according to the invention, when it is present in a lactic acid bacterial strain does not cause any substantial growth inhibition of the host strain. Thus, the vector, when it is present in the *Lactococcus lactis* strain CHCC4146 (DSM 12109), permits this strain to acidify milk at essentially the same rate as that of the same strain not containing an amber suppressor.

The term "milk" as used herein is intended to mean any type of milk or milkcomponent which does not contain the precursors for the synthesis of pyrimidine nucleotides including e.g. cow's milk, human milk, buffalo milk, goat's milk, sheep's milk, or whey.

Evidently, the above-mentioned acidification of milk will result in essentially the same pH decrease in the medium inoculated with the respective strains. However, it is contemplated that with other host strains, the acidification rate may be insignificantly reduced by the presence of the vector according to the invention. Accordingly, the expression "at essentially the same acidification rate" includes that in comparative acidification experiments, the ΔpH after about 3 hours of cultivation is at the most 1.0 such as at the most 0.5 including at the most 0.2 such as e.g. at the most 0.1.

It is another objective of the invention to provide recombinant food-grade vectors that do no inhibit growth rate of lactic bacterial host cells. Thus, as it is also shown in the below examples, the vector of the present invention permits a host cell such as *Lactococcus lactis*

FA4-1-1 strain to grow at 30°C in minimal medium at a doubling time which is shorter than that for the strain DN209 transformed with the vector pFG1.1 (DSM 12088). In useful embodiments the doubling time of a host cell transformed with the vector of the invention is at least 5 minutes shorter than that of the strain DN209 transformed with the vector pFG1.1, such as at least 10 minutes shorter e.g. at least 15 minutes shorter or even at least 20 minutes shorter.

In accordance with the invention, the recombinant vector has at least one of the above characteristics (i) to (iii). However, it is preferred that the vector has at least two of such characteristics and most preferably all of these characteristics.

The suppressor gene comprised in the recombinant vector is typically derived from a lactic acid bacterial cell which is subjected to a mutagenisation treatment followed by selecting mutants suppressing an amber nonsense mutation e.g. such as it is described by Dickely et al. 1995 and isolating a DNA sequence comprising the mutated suppressor gene. However, the suppressor gene comprised in the recombinant vector can be provided by selecting a spontaneously occurring mutant in accordance with the screening method as described above.

Alternatively, it is possible to construct a tRNA gene coding for the suppressor by conventional DNA synthesis methods or by *in vivo* mutagenesis of isolated genes. Normally, the mutated tRNA-encoding gene is derived from the chromosome of the source strain. Preferably, the suppressor gene encodes a tRNA with an anticodon selectively recognising amber codons, i.e. an amber suppressor. The nonsense suppressor may be one which results from at least one nucleotide change in a gene coding for a tRNA anticodon resulting in the altered tRNA anticodon CUA. In useful embodiments the suppressor is a *supD, supE, supF, supP, supU* or a *supZ* suppressor.

In other specific embodiments, the amber suppressor may be derived by double or triple base substitutions in the anticodon region of the tRNA.

The DNA sequence comprising the tRNA encoding suppressor gene is preferably a small sequence such as a sequence in the range of 0.05 to 10 kb, more preferably in the range of 0.1 to 5.1 kb, such as e.g. 3.2, 1.1 or 0.25 kb. As an example, the DNA sequence coding for such a tRNA may be the following (SEQ ID NO:1):

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1    GGAGCCATGG CAGAGTGGTA ATGCAACGGA CTCTAAATCC GTCGAACCGT
51   GTAAAGCGGC GCAGGGGTTC AAATCCCCTT GACTCCTTA
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In one interesting embodiment of the present invention, the vector is one wherein the gene coding for a nonsense suppressor is under the control of a regulatable promoter. As used herein, the term "regulatable promoter" is used to describe a promoter sequence possibly including regulatory sequences for the promoter, which promoter is regulatable by one or more factors occurring during the growth of a host cell comprising the recombinant vector. Such factors include the pH and/or the arginine content of the growth medium, the growth temperature, a temperature shift eliciting the expression of heat shock genes, the composition of the growth medium including the ionic strength/NaCl content and the growth phase/growth rate of the lactic acid bacterium. Such a regulatable promoter may be the native promoter or it may be an inserted promoter not naturally related to the suppressor gene either isolated from the lactic acid bacterial species or it may be a heterologous promoter sequence, i.e. a sequence derived from a different lactic acid bacterial species.

A promoter sequence as defined above may comprise further sequences whereby the promoter becomes regulated by a stochastic event. Such a regulation may e.g. be useful in lactic acid bacterial cultures for which it may be advantageous to have a gradually decreasing activity of the suppressor gene under control of the promoter sequence. Such further sequences may e.g. be sequences, the presence of which results in a recombinational excision of the promoter or of genes coding for substances which are positively needed for the promoter function.

In accordance with the present invention, the vector is typically constructed by combining a DNA sequence comprising a suppressor gene which is functional in a lactic acid bacterium and a replicon capable of replicating in a lactic acid bacterium. In a useful embodiment the replicon is from a *Lactococcus lactis* plasmid. More specifically, the replicon is derived from the *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* citrate plasmid pCT1138 or the *Lactococcus lactis* plasmid pIL2608 such as it is described in the following examples.

For a vector construct as described above to be useful as a cloning vector it is provided with at least one restriction site. Preferably, the restriction site(s) is/are unique sites. In useful embodiments, the vector comprises at least one DNA sequence containing multiple cloning sites.

Examples of vectors, which are encompassed by the present invention, are the multi-copy vectors pFG100 and pFG200 as described in the examples.

In addition, the present invention encompasses mutants, variants or derivatives having essentially the characteristics of the vectors pFG100 and pFG200. In this context, the terms "mutant", "variant" or "derivative" refers to any modification of the DNA sequence of the above vectors including substitution, addition or deletion of nucleotides in the suppressor gene, the replicon, any regulatory DNA sequences or any other sequence of the vector, that substantially does not affect the characteristics of the modified vector, relative to the parent vector.

Preferably, vectors as described above have a size allowing for the insertion of desirable genes. Accordingly, a suitably sized vector as defined herein has a size which is in the range of 0.5 to 20 kb, although larger vectors may also be used. In preferred embodiments the vector has a size in the range of 1 to 10 kb, such as in the range of 2 to 5 kb.

The vector according to the invention may further comprise an inserted gene coding for a desired gene product. In this context, interesting desired gene products include genes coding for enzymes which have an advantageous effect on the quality of a food product, the manufacturing or preservation of which includes the addition of viable lactic acid bacterial cultures as it has been described above. Thus, such genes inserted into the vector may code for a peptidase, including lysine-aminopeptidase, glutamyl-aminopeptidase, cysteine-aminopeptidase, iminopeptidase, X-prolyl-dipeptidyl aminopeptidase, endopeptidase, dipeptidase or tripeptidase.

In the present context, other interesting gene products include lipases, proteases, nucleases and enzymes which are involved in the carbohydrate metabolism of the host bacterium. Inserted genes may also be prokaryotic or eucaryotic genes isolated from non-lactic acid bacterial species. Additionally, one useful gene product includes a gene product which is involved in nisin synthesis or nisin resistance.

In accordance with the invention, an interesting gene product includes a gene product conferring bacteriophage resistance to the lactic acid bacterial host cell. In another useful embodiment the vector according to the invention comprises a gene coding for a bacteriophage lysine, which in a specific embodiment is derived from the bacteriophage

ØvML3 contained in the strain DN209/pFG7 deposited with the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg, 1b, D - 38124 Braunschweig on 6 April 1998 under the accession No. DSM 12089. In a further specific embodiment the vector according to the invention is a theta-replicating vector.

As it mentioned above, it is a further object of the invention to provide food-grade vectors which can be stably maintained in a lactic acid bacterial host cell. Accordingly, there is preferably substantially no loss of the vector when the host strain is grown for at least 20 generations, including at least 35 generations such as at least 50 generations or even at least 100 generations in a medium wherein a host cell not containing the vector is not capable of growing. Thus, when the host cell comprises a nonsense mutation conferring auxotrophy e.g. with respect to amino acid or nucleotide precursor compounds, such as purine or pyrimidine precursors, the vector will be selected (stabilised) in a medium not containing such precursors.

The invention provides in a further aspect a lactic acid bacterium comprising a vector according to the invention. Normally, the bacterium also comprises an amber mutation being suppressible by the nonsense amber suppressor. Such a gene coding for the nonsense mutation may be located on a replicon different from the one containing the gene coding for a nonsense suppressor, e.g. on the chromosome, on a plasmid or it may be incorporated in the cell as a prophage.

In certain preferred embodiments, the lactic acid bacterium of the present invention comprises a suppressor which is capable of suppressing a nonsense mutation which, in the absence of the nonsense suppressor, confers auxotrophy. Such a nonsense mutation may e.g. be in a gene involved in the synthesis of pyrimidine nucleotides from their precursors in which case the lactic acid bacterium is a nonsense *pyr* mutant such as a *pyrF*$^-$ mutant.

The lactic acid bacterium of the invention can be any lactic acid bacterium selected from the group consisting of a *Lactococcus* sp., *Streptococcus* sp., *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Bifidobacterium* sp. One preferred species is *Lactococcus lactis* including *Lactococcus lactis* subsp. *lactis*. Examples of strains belonging to the latter species include strain FA4-1-1 containing pFG100, deposited under

the accession No. DSM 12091 and *Lactococcus lactis* subsp. *lactis* strain CHCC4146 containing pFG200, deposited under the accession No. DSM 12108.

In a still further aspect, the invention relates to an isolated pure culture of a lactic acid bacterium as defined above, the expression "pure culture" indicating that the culture contains a biomass of one single isolate of a lactic acid bacterial species, i.e. a clone originating in principle from one cell. Such a pure culture may be provided as a liquid cell suspension or as frozen or freeze-dried preparation. Preferably the pure culture is in a concentrate of cells obtained by separation e.g. by centrifugation or filtration using conventional techniques.

In yet a further aspect, the present invention relates to a composition comprising an isolated pure culture of a lactic acid bacterium as defined above, and a micro-biologically acceptable carrier. It may be preferred that such a composition contains at least 10^5 colony forming units (CFUs) of the bacterium such as at least 10^7 or at least 10^9 CFUs per g. Suitable carriers substances include nutrients such as an assimilable carbohydrate or a nitrogen source, which can be utilised readily by the lactic acid bacterium. Typically, such a composition is provided in the form of a frozen or freeze-dried composition. In the latter case, the composition may contain cryoprotective compounds.

The composition may, in accordance with the invention, comprise two or more different species of lactic acid bacteria or two or more strains of the same species. It is common in the production of food products, where lactic acid bacterial starter cultures are used, to apply mixed cultures, i.e. cultures comprising a multiplicity of strains. As an example hereof it can be mentioned that a mixed culture of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* is typically used in the production of yoghurt. In other dairy products a mixed culture of *Bifidobacterium bifidum* and *Lactobacillus acidophilus* are used.

In further aspects, the invention relates to the use of the above composition as a starter culture in the preparation of a food product such as e.g. a dairy product, a vegetable product, a meat product, a bakery product or a wine product, and its use in the production of an animal feed such as silage, from e.g. grass, cereal, peas, alfalfa or sugar-beet leaf, where starter cultures are inoculated in the feed crop to be ensiled in order to obtain a preservation hereof, or in protein rich animal waste products such as slaughtering offal and fish offal, also with the aims of preserving this offal for animal feeding purposes. Yet

another significant application of lactic acid bacterial cultures according to the present invention is the use of such cultures as so-called probiotics. By the term "probiotic" is in the present context understood a microbial culture which, when ingested in the form of viable cells by humans or animals, confers an improved health condition, e.g. by suppressing harmful microorganisms in the gastrointestinal tract, by enhancing the immune system or by contributing to the digestion of nutrients.

In another specific embodiment, the above composition is used as a starter culture in the preparation of a dairy flavour used for e.g. flavouring of butter, margarine, spreads, cereal products or pop-corn, or for a product for cheese flavouring.

In accordance with the present invention there is also provided a method of stably maintaining a vector of the present invention in lactic acid bacterial host cells growing in a particular environment, comprising providing said host cells as nonsense mutant cells having lost the capability of growing in said environment, and transformed with a vector according to the invention containing a nonsense suppressor gene encoding a gene product restoring the capability of the nonsense mutant cells to grow in said environment whereby, if the vector is lost from the lactic acid bacterial cells, the cells will not grow.

In suitable embodiments of the invention, the lactic acid bacterial host cells harbouring the vector to be stably maintained have a nonsense mutation in one or more genes conferring auxotrophy to the cells whereby the cells have lost their capability to grow in the particular environment due to a lack herein of an essential nutrient substance which cannot be synthesised by the nonsense mutant cells.

As one example, the nonsense mutation may be one which causes the host cells to lose the capability to grow in a medium which does not contain pyrimidines.

Accordingly, the suppressor gene of the vector functions as a selective marker for the lactic acid bacterial host cells. In the present context, the term "a selective marker" is used to designate a gene coding for a product which renders lactic acid bacterial cells unable to grow if the vector to be maintained is lost from the cells.

Thus, in accordance with the invention, auxothrophic nonsense mutants may be isolated, which allow a vector to be stably maintained in a lactic acid bacterium growing in specific

environments including milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough, a batter, the gastrointestinal tract, feed crops or offal to be ensiled by a lactic acid bacterium.

The invention is further illustrated in the following non-limiting examples and the drawings wherein

Fig. 1 illustrates the construction of vector pFG100. Ligation of a 2.8 kbp *Eco*RI-*Bam*HI DNA fragment carrying the replicon of vector pIL2608 and a 298 bp PCR fragment containing *supD* suppressor allele;

Fig. 2 shows the growth rate of strain FA4-1-1 harbouring pFG100 compared to the growth rate of strain DN209 harbouring pFG1.1;

Fig. 3 shows the growth rates of the mother strain MG1363, DN209 harbouring pFG1.1, FA 4-1-1 harbouring pFG100 and FA 4-1-1 harbouring pFG200 (each determined twice);

Fig. 4 illustrates the construction of vector pMPJ100. Ligation of a 2.8 kbp *Eco*RI DNA fragment carrying the replicon of plasmid pIL2608 and a 298 bp PCR fragment containing *supD* suppressor allele. Filled-in arrows indicate the deletion of the non food-grade part by *Bam*HI digestion of pMPJ100 to generate the food-grade vector pFG100. Only unique cloning sites in pMPJ100 are shown (except *Bam*HI);

Fig. 5 illustrates the construction of vector pFG200. Ligation of an *Eco*RI digestion of plasmid pKR41 carrying a replicon and an *Eco*RI digestion of pAK93 carrying the amber suppressor resulting in the plasmid pMPJ103. *Hind*III-digestion of pMPJ103, self-ligation and electroporation of strain FA4-1-1, resulting in vector pFG200;

Fig. 6 shows the pH development during 16 hours in whole milk inoculated with strain FH CY-1 or strain CHCC4171 (FH CY-1 $pyrF_{amber}$ containing pFG100);

Fig. 7 shows the pH development during 16 hours in whole milk inoculated with strain FH CY-1 or strain CHCC4223 (FH CY-1 $pyrF_{amber}$containing pFG200);

Fig. 8 shows the DNA sequence of the *pyrF* gene of FH CY-1(SEQ ID NO:23 and SEQ ID NO24);

Fig. 9 illustrates the construction of a $pyrF_{amber}$ mutation; and

Fig. 10 illustrates the introduction of the $pyrF_{amber}$ mutation into the host strains chromosome.

EXAMPLES

Materials and Methods for the construction of the food-grade vectors pFG100, pFG101 and pFG200

(i) Bacterial strains and plasmids

The following *Lactococcus lactis* strains were used in the examples: strain MG1363 is a plasmid-free derivative of *Lactococcus lactis* strain NCDO 712 (Gasson, 1983). Strain FA4-1-1 deposited on 6 April 1998 under the accession No. DSM 12086 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany having a nonsense mutation that is a pyrimidine auxotroph (pyrF) of strain MG1363, suppressible by an amber suppressor. Strain CHCC4146 deposited on 17 April 1998 under the accession No. DSM 12109 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany is a pyrimidine auxotroph (pyrF) of strain FH CY-1 having a nonsense mutation that is suppressible by an amber suppressor.

The following plasmids were used as sources for replicons: *Lactococcus lactis* plasmid pIL2608 (INRA, France), and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* citrate plasmid pCT1138.

(ii) Growth media and conditions

When strain MG1363 was grown in liquid medium, M17 with 0.5% glucose (GM17) was used. Strain FA4-1-1 or other *pyrF*-derivatives were cultivated in GM17 medium or DN

minimal medium (Dickely et. al. 1995) with 40 µg/ml uracil and/or 40µg/ml thymidine added. Solid medium (agar plates) was made by the addition of 1.5% agar to the liquid medium. Selection and maintenance of vectors carrying the *supD* allele in *pyrF*⁻ strains were performed in minimal medium without pyrimidine sources. Selection of plasmid pMPJ100 was also carried out in media containing 10g tetracycline. The growth temperature was 30°C. Exponential growth on minimal medium was measured by monitoring the increase in OD at 600 nm.

(iii) Preparation of competent cells

Competent cells of strain MG1363 and derivatives were prepared by growing the cells in rich medium containing 1% glycine and for FH CY-1 derivatives 1.5% glysin (Holo and Nes, 1989). Pyrimidine requiring strains were grown in medium containing 40µg/ml uracil and/or 40µg/ml thymidine to reduce the frequency of revertants. Cells were harvested at an OD_{600} of 0.6-0.8, resuspended in 0.5M sucrose/10% glycerol and then frozen at -80°C.

(iv) Electroporation of glycine-grown competent cells

The conditions for electroporation were in all experiments: 25 µF, 200 W, 2.0 kV. Using these conditions with desalted DNA, the typical time constant was 4.8 (Holo and Nes, 1989).

(v) Plasmid purification

Purification of plasmids from lactococcal strains was carried out as described by Pedersen et al. 1994. Larger preparations of plasmid DNA were prepared using a plasmid purification kit as described by the supplier (QIAGEN®, Stratagene®, Genomed®) by including an initial step of lysozyme treatment. Plasmid DNA preparations were kept at 4°C in 10 mM Tris (pH 7.5).

(vi) PCR-reactions

PCR-amplification of the *supD* gene was performed using 50 ng of pAK89 as template, 10-50 pmol of each primer, 0.25 mM of dNTP, 0.5 units of Taq enzyme in diluted (1x) buffer,

which is supplied with the enzyme. The total volume was 50 µl. The reaction conditions were: 94°C for 4 min, 35 cycles of 94°C, 1 min; 50°C, 1 min, 72°C; finally 72°C for 7 min.

(vii) Agarose gel electrophoresis

Agarose gel electrophoresis was used for verification of purified DNA, digested DNA, ligated DNA or for further purification of separated DNA fragments. To separate DNA fragments above 500 bp, DNA was loaded and electrophoresed through an agarose gel made with 1% agarose in a Tris-Borate, EDTA buffer. To test and separate DNA fragments less than 500 bp in size, a gel with a higher agarose content was used (1.5-2%). The electrophoresis was performed by applying 100V for 45-60 minutes using a Biorad Power supply Model 200, 2.0.

EXAMPLE 1

The construction of the food-grade vector pFG100

1.1 Introduction

In order to use genetically manipulated microorganisms in food products, vectors that are derived totally from the organism to be manipulated are desirable. A useful vector contains a replication region, a selectable marker and a multiple cloning site allowing insertion of desirable genes. In addition, it should be small enough to allow insertion of desired DNA without difficulty.

A multi-copy food-grade cloning vector, pFG100 replicating in lactic acid bacteria was constructed which is based totally on DNA sequences from *Lactococcus*. pFG100 contains the replication region of the *Lactococcus lactis* plasmid pIL2608, an amber suppressor encoding gene, *supD*, and a multiple cloning site (SEQ ID NO:25 and SEQ ID NO:26). The plasmid is present in >5 copies per cell and exhibits a stable phenotype in various Lactococcal strains.

1.2 Construction of pFG100 by combining the replication region with the suppressor gene

Vector pFG100 was constructed by ligation of a 2.8 kbp *Eco*RI-*Bam*HI DNA fragment carrying the replicon of the plasmid pIL2608 to a 298 bp PCR fragment containing the *supD* suppressor allele. This PCR fragment was generated with plasmid pAK89 (Dickely et al. 1995) as template by using the following primers:

amber-3 (SEQ ID NO:2): (CGAATTCATATTTGATTAATGAGAATATGGAACC)
amber-4 (SEQ ID NO:3): (CGGGATCCTTTCAGGAAGGTAATTAAC)

The primers are complementary to the promoter region and downstream region of *supD* (Fig. 1). The primers were carrying *Eco*RI and *Bam*HI linkers respectively, which after digestion gave ends compatible for ligation to the replicon fragment of pIL2608.

1.3 Selection of pFG100

As the primary bacterial host strain for the ligated DNA, strain MG1363 was chosen as this strain can be made more competent than the *pyrF* strain FA4-1-1.

Since strain MG1363 does not carry a stop-codon in the *pyrF* gene, this strain cannot be used for direct selection of pFG100. In order to select for pFG100, strain MG1363 was co-transformed with pFDi3 (Dickely et. al. 1995), which carries an amber stop-codon in the gene coding for erythromycin resistance. Using this approach a total of 18 colonies were obtained on GM17 agar plates containing 1μg/ml erythromycin. No colonies appeared on the control plate (MG1363/pIL2608+pFDi3). Eight of the colonies were streaked to single colonies for purification and after plasmid preparation, one clone harbouring pFDi3 and pFG100 was selected. After agarose gel electrophoresis, vector pFG100 was cut out from the gel.

1.4 Transformation of the bacterial strain FA4-1-1

The isolated pFG100 vector was introduced into FA4-1-1 by electroporation of glycine-grown competent cells to transform FA4-1-1 to pyrimidine prototrophy. The strain FA4-1-1 containing the vector pFG100 was deposited on 6 April 1998 under the accession No. DSM

12091 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany.

The electroporation efficiency with pFG100 into FA4-1-1 is highly variable. To obtain good competent cells of FA4-1-1, it is recommended that the strain is grown exponentially in the presence of a pyrimidine source as described above. The OD at 600 nm should not exceed 0.7. Using these conditions, good competent cells were obtained regularly. The typical electroporation efficiency with pFG100 DNA has been calculated to 1-5x10^5 colonies per µg DNA. For direct electroporation of small amounts of ligated DNA, FA4-1-1 was found to be unsuitable as host.

1.5 Growth and stability of bacterial strains harbouring pFG100

pFG100 can be transformed into the bacterial host strain FA4-1-1 with relatively good efficiency and then isolated with reproducible result. Plasmid isolations from FA4-1-1 tend to yield chromosomal DNA to a higher level than usual.

The growth rate of FA4-1-1 harbouring pFG100 has been measured and compared to the growth rate of DN209/pFG1.1 deposited on 6 April 1998 under the accession No. DSM 12088 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany (Table 1 and Fig. 2). Vector pFG1.1 is a pFG1 derivative (Dickely et. al. 1995) with a mutation in the *supB* promoter, which reduces *supB* expression and allows the host strain to grow faster.

Table 1. Growth rate of strain FA4-1-1 harbouring pFG100 compared to the growth rate of strain DN209 harbouring pFG1.1. on minimal medium

Time	pFG1.1	pFG100
12:01	0.20	0.21
12:50	0.27	0.30
13:48	0.37	0.57
15:05	0.61	1.04
16:00	0.84	1.41

FA4-1-1/pFG100 was found to grow with a doubling rate of 86 minutes which is 19 min faster than DN209/pFG1.1 (105 minutes) (Fig. 2).

In a comparative study of the growth rates of the parental strain MG1363, strain DN209/pFG1.1, strain FA 4-1-1/pFG100 and strain FA 4-1-1/pFG200 (Table 2, Fig. 3), the strains were found to grow with a doubling rate of 66 min, 115 min, 77 min and 77 min, respectively.

Table 2. Growth rates of strain MG1363, strain DN209/pFG1.1, strain FA 4-1-1/pFG100 and strain FA 4-1-1/pFG200 on minimal medium

Time (min)	**MG1363**	**pFG1.1**	**pFG100**	**pFG200**
0	0,063	0,063	0,067	0,058
71	0,094	0,081	0,079	0,076
149	0,190	0,134	0,172	0,162
196	0,304	0,204	0,245	0,235
276	0,660	0,318	0,467	0,451
339	1,117	0,410	0,770	0,737
423	1,615	0,544	1,200	1,182
	Td=66min	Td=115min	Td=77min	Td=77min

1.6 Stability of pFG100 in industrial production strains

To test the stability of pFG100 in production strains, the vector was transformed into the *pyrF*-derivative of strain FH CY-1, strain CHCC4146 to produce strain CHCC4171 which was deposited on 6 April 1998 under the accession No. DSM 12090 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany. The vector was found to be maintained and co-exist with the plasmids of that strain. Importantly, it was also found that growth and lactic acid production was virtually unaffected by the presence of the vector (see Example 4).

1.7 Plasmid copy number of pFG100

The copy number of pFG100 was determined by visual comparison by agarose gel electrophoresis with the vector pFG1 which was deposited on 6 May 1994 under the accession No. DSM 9190 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany. These data indicate that pFG100 is present in 5-8 copies per cell corresponding to the copy number of vector pFG1.

EXAMPLE 2

The construction of the non-food grade vector pMJP100 as an intermediate to obtain the food-grade vector pFG101

2.1 Introduction

The primary host strain FA4-1-1 exhibits a quite high reversion rate and is difficult to make competent enough for electroporation with ligation mixtures i.e. initial screenings of clones. To circumvent this problem, the pMPJ100 vector was constructed. Like pFG100, this vector is also based on the pIL2608 replicon but in addition to the *supD* gene, the *tet* gene is present on the plasmid. This means that the cloning of relevant genes can be screened in MG1363 by selecting for tetracycline resistance. Conversion to food-grade status can then be carried out by a *Bam*HI digestion, ligation and transformation into the final *pyrF* amber host.

2.2 Construction of plasmid pMPJ100 by combining the replicon region with the suppressor gene

Plasmid pMPJ100 was constructed by digestion of plasmid pIL2608 with *Eco*RI and the DNA with a PCR fragment carrying the *supD* allele (Fig. 4). This PCR fragment was generated with the plasmid pAK89 (Dickely et. al. 1995) as described in Example 1, but instead of using primer amber-4 (SEQ ID NO:3), the following primer was used to obtain *Eco*RI compatible sites in both ends of the fragment:

primer amber-6 (SEQ ID NO: 5) (GGGAATTCAGGAAGGTAATTAACTATGG)

2.3 Transformation of the bacterial strain MG1363 with pMJP100

The ligation mixture was introduced into MG1363 by electroporation. Electroporation with ½ of the ligation mixture into MG1363 resulted in more than 300 colonies on GM17 agar plates containing 10 tetracycline. Ten colonies from this plate were further purified and used for isolation of plasmid DNA. Two of the 10 colonies were found to contain the *supD* allele cloned in the same orientation (Fig. 4).

The electroporation efficiency with pMPJ100 DNA is typically the same as with pFG100, as described in Example 1. The electroporation efficiency has been calculated to 1-5 x 10^5 colonies per µg DNA.

2.4 Deletion of the nonfood-grade components of pMJP100 to obtain the food-grade vector pFG101

Food-grade vector pFG101 can be derived directly from pMPJ100 by *Bam*HI digestion, ligation and transformation into a *pyrF*-$_{amber}$ host strain (Fig. 4).

EXAMPLE 3

Construction of the food-grade expression vector pFG200

The complete minimal replicon of the *L. lactis* subsp. *lactis* biovar *diacetylactis* citrate plasmid pCT1138 has been cloned as a 1.7 kb polymerase chain reaction (PCR) fragment flanked by *Eco*RI sites (Pedersen et al., 1994). This fragment contains the origin of replication, the *repB* gene and ~300bp of flanking DNA and was cloned in pIC19H (ampicillin resistant, Amp^R) to produce pKR41.

PCR was performed on pAK89 using the following primers:

amber-2 (SEQ ID NO:4): (CGAATTCAACATTTTTGTATAAATATGCG)
amber-3 (SEQ ID NO:2): (CGAATTCATATTTGATTAATGAGAATATGGAACC)

The primers were used to produce a PCR fragment containing the tRNA promoter, the suppressor gene and downstream sequences flanked by the *Eco*RI sites provided by the primers (Fig. 5). This 360 bp *Eco*RI fragment was cloned in pIC19H to give pAK93.

The *supD* allele is a suitable selectable marker when combined with the pyrimidine auxotroph FA4-1-1. This strain only grows in pyrimidine-free medium in the presence of the amber suppressor. DNA of pAK93 and pKR41 was digested with *Eco*RI, mixed, ligated and electroporated into FA4-1-1 followed by selecting prototrophs. This selection ensures that colonies contain plasmids with the suppressor gene and the citrate plasmid replicon. Some plasmids will also contain pIC19H. These were obtained by pooling several hundred colonies, extracting plasmid DNA and transforming *E. coli* selecting Amp^R. One plasmid with the desired structure was designated pMPJ103.

All pIC19H except the polylinker was deleted by digesting pMPJ103 with *Hind*III, self ligating, and electroporating FA4-1-1 selecting prototrophs. Among 300 colonies obtained in total, 20 were selected and tested for plasmid content. More than 50% of these was found to contain a single plasmid of 2.2 kb containing the citrate plasmid replicon, the amber suppressor and the polylinker. One was saved and the plasmid designated pFG200. The vector pFG200 was transformed into the *pyrF*-derivative of strain FH CY-1, strain CHCC4146 to produce strain CHCC4223 which was deposited on 16 April 1998 under the accession No. DSM 12108 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany. The structure of pFG200 is illustrated in Fig. 5. The resulting polylinker (SEQ ID NO:27) is identical to that found in pIC19R (Marsh et al., 1984) and contains the following unique sites: *Sma*I, *Bam*HI, *Sal*I, *Pst*I, *Hind*III, *Nru*I, *Xho*I, *Sac*I, *Bgl*I, *Xba*I and *Eco*RV.

EXAMPLE 4

Construction of $pyrF_{amber}$ mutants of the industrial strain *Lactococcus lactis* FH CY-1

4.1 Introduction

In order to use the previously described food-grade expression vectors based on a *supD* suppressor capable of suppressing amber mutations, appropriate mutations in a number of

industrial *Lactococcus* strains are needed. Rather than use chemical mutagenesis with its inherent problems, single precise alteration in the chromosome of the strains to be used as host for pFG100 or pFG200 were carried out. The *pyrF* gene was chosen because it exists in a single copy in the *Lactococcus* chromosome, the DNA sequence is known from MG1363, mutants should have an absolute requirement for pyrimidines for growth, and milk does not contain enough pyrimidines to allow growth of the mutants. Thus, milk would be a selective medium for this plasmid.

The DNA sequence of the *pyrF* gene of FH CY-1 was determined, the *pyrF* genes was cloned and amber mutations introduced by polymerase chain reaction. Finally, gene replacement was used to introduce the constructed amber mutation into the chromosome of FH CY-1.

4.2 Materials and methods

(i) Growth media and strains

Lactococcus lactis was grown in M17 or DN minimal medium (Dickely et al., 1995). *Escherichia coli* was grown in LB-media, supplemented with ampicillin to 50 mg/ml when required. For growth of *pyr*⁻ strains, uracil was added to 20 mg/ml. Competent cells of FH CY-1 were made by growth in the presence of 1.5% glycine (Holo and Nes, 1989).

Strain FH CY-1, which was deposited on 6 April 1998 under the accession No. DSM 12087 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany, is the industrial strain of *Lactococcus lactis* also known as CHCC377 and is the major component of the R604 culture. *Escherichia coli* strain DH5α was used for cloning in pIC19H.

(ii) DNA preparation and manipulations

Plasmid preparations from *Escherichia coli* was prepared by using a plasmid purification kit as described by the supplier (QIAGEN®, Stratagene®, Genomed®) by including an initial step of lysozyme treatment. Plasmid DNA was isolated *from Lactococcus lactis* by using the procedure of Pedersen et al. (1994) with the modification that 50ml of 5M NaCl was

added before the phenol extraction. Chromosomal DNA was purified from *Lactococcus lactis* by using the procedure of Johansen and Kibenich (1992).

Digestion with restriction enzymes, ligations and polymerase chain reactions were made by following the procedure of the manufacturers of the various enzymes and kits. DNA sequencing was by cycle sequencing as recommended by Perkin-Elmer. The resulting products were purified on BioRad Micro-Bio-Spin-Chromatography columns before running on the ABI310 DNA sequencer. The primers used are shown in Table 3.

Table 3. Primer used

pyrF1	SEQ ID NO:6	GCAGATCTAAGCTTGATTCAAGAAGTAAAAGAAGGC
pyrF2	SEQ ID NO:7	ATAGATCTACTCGATGCCAAGAATGGACCGC
pyrF3	SEQ ID NO:8	AAAGGCCTGTNATNGCNCTNGAYTTYCC
pyrF4	SEQ ID NO:9	TGGACGAATTCCNGGNGT
pyrF5	SEQ ID NO:10	CATAGTAAACGACTTGGGG
pyrF6	SEQ ID NO:11	TACGCACAAAAAACCGCT
pyrF7	SEQ ID NO:12	GGTCGCCTTTACTTGCACC
pyrF8	SEQ ID NO:13	GATTATATTGTTGTCGGCCG
pyrD-degn	SEQ ID NO:14	GCTCTAGAGCMWATYGWWATDGGN
llagidB2	SEQ ID NO:15	GGTNGARTGGAAYGARAARATHAAY
Fam5	SEQ ID NO:16	CCTCAACCTAGGAGAAAATTATGC
Fam6	SEQ ID NO:17	TCTCCTAGGTTGAGGTTAATTGTG
pyrD/BglII	SEQ ID NO:18	ATAGATCTGCTTAGAAAACTTG
pyrF11/BglI	SEQ ID NO:19	ATAGATCTGCATGTAAGCAAAAACC

(iii) Plasmid stability in milk

A fresh overnight culture of CHCC4171 (DSM 12090) and CHCC4223 (DSM12108) in minimal medium was subcultured 1:100 (100µL into 10 ml) in reconstituted skim milk (RSM) and incubated overnight. The resulting culture was plated on M17 plates and subcultured 1:100 in RSM. This procedure was repeated for five consecutive days. Each outgrowth was taken to be 7 generations (2^7=128). After each outgrowth, 100 single colonies were picked and patched onto minimal and minimal + uracil plates. A strain which

has lost the pFG100 or pFG200 plasmid will be unable to grow on minimal plates but will grow on minimal + uracil plates. A strain retaining the plasmid will grow on both plates. To confirm this, plasmids were isolated from 10 colonies after 35 generations. All had the expected plasmid profile.

(v) Acidification studies in whole milk

Single colonies of strain FH CY-1 (DSM 12087), and CHCC4171 (FH CY-1 $pyrF_{amber}$/pFG100), DSM 12090) were inoculated in M-17 or DM-9,5-UA media. The cultures were incubated for 24 hours at 30°C. Subsequently, the cultures (1%) were inoculated in whole milk and incubated in a warm water-bath (Profilur) equipped with a pH-electrode (Hammilton). The pH development was registered continuously for 16 hours (Fig 6). In addition, comparative acidification studies with strain FH CY-1 and CHCC4223 (FH CY-1 $pyrF_{amber}$/pFG200), DSM 12108) were performed in accordance with the method as described above (Fig. 7).

4.3 Sequencing of the *pyr*F gene of strain FH CY-1

The DNA sequence of the *pyr*F gene of MG1363 is known (Andersen et al., 1994). Primers based on this sequence (pyrF1 and pyrF2, Tab. 3) were tested but could not be used to clone or sequence the *pyrF* gene of FH CY-1. Based on an amino acid sequence comparison of the *pyrF* gene product of a number of microorganisms, two degenerate primers (pyrF3 and pyrF4, Tab.3) were made and used to amplify a 550 bp internal *pyrF* fragment. This fragment was sequenced with the same primers. Based on this sequence, new primers were ordered (pyrF5 and pyrF6, Tab. 3) and used for inverse PCR with *Sau*3AI digested DNA. This gave additional sequence data. The sequence of the amino terminal end of *pyrF* was completed using a degenerate primer designed from the sequence of *pyrD* (pyrD-dgen, Tab. 3) which is immediately upstream of *pyrF* and two additional primers (pyrF7 and pyrF8, Tab. 3). The carboxy terminal end of *pyrF* was sequenced using a degenerate primer (IlagidB2, Tab. 3) designed from the sequence of *gidB*, which is immediately downstream of *pyrF*, and pyrF6. The DNA sequence of the *pyrF* gene of FH CY-1 is presented in Fig. 8. This gene is 86% identical to the *pyrF* gene of MG1363.

4.4 Cloning of the *pyrF* gene of strain FH CY-1

Primers for PCR amplification were designed from the DNA sequences determined above (pyrD/BglII and pyrF11/BglII). They contain *Bgl*II restriction sites and allow amplification of a ca. 1.1 kb DNA fragment containing the entire *pyrF* gene. This fragment was cloned into pIC19H digested with *Bgl*II. The *pyrF* gene of strain FH CY-1 is contained in a plasmid designated pAK142.

4.5 Introduction of an amber mutation into the cloned *pyrF* genes

The strategy used involves searching the DNA sequence for a serine codon (TCT, TCC, TCA, TCG, AGT or AGC) that can be changed to amber (TAG) and which is flanked by sequences that allow the introduction of a restriction enzyme recognition site without affecting the amino acids encoded by the flanking sequences. This is illustrated below:

```
Fam5                    5' CC TCA ACC TAG GAG AAA ATT ATG C 3' ->
                           || ||| ||* |** ||| ||| ||| ||| |
     5'  ... ACA CAA TTA ACC TCA ACT TCT GAG AAA ATT ATG CAA ... 3'

pyrF          T   Q   L   T   S   T   S   E   K   I   M   Q

     3'  ... TGT GTT AAT TGG AGT TGA AGA CTC TTT TAA TAC GTT ... 5'
               | ||| ||| ||| ||| ||* |** ||| |
 Fam6      <-3' T GTT AAT TGG AGT TGG ATC CTC T 5'
```

The PCR primers are called Fam5 and Fam6 (Tab. 3) and are indicated above and below the sequence of this region of *pyrF* (SEQ ID NO:20 and SEQ ID NO:21). The mismatched base pairs are indicated by *. The outside primers are pyrF11/BglII and pyrD/BglII (Tab. 3) to facilitate cloning of the resulting PCR products. Following PCR and cloning as described in Figure 9, the DNA sequence (SEQ ID NO:22) will be:

```
                                        amber
     5'     ... ACA CAA TTA ACC TCA ACC TAG GAG AAA ATT ATG CAA ... 3'

                T   Q   L   T   S   T   ¤
```

where the amber codon is indicated by ¤ and the introduced *Avr*II restriction site is underlined. Suppression of this amber mutation by *supB* will introduce a serine and the amino acid sequence of the resulting protein will be identical to that of the parent strain.

The *pyrF*$_{amber}$ DNA fragment was cloned in pIC19H to make plasmid pAK148 and the DNA sequence determined. No undesired changes were detected.

4.6 Introduction of the amber mutation into the chromosome

The *pyrF*$_{amber}$ DNA fragment was subcloned into pG$^+$Host9 to produce plasmid pAK149. The pG$^+$Host vectors are temperature sensitive for replication and can be used to integrate DNA into the *Lactococcus* chromosome (Fig. 10). These vectors replicate at 30°C but not at temperatures above 36°C. The strain FH CY-1/pAK149 was constructed by electroporation and is resistant to erythromycin by virtue of the erythromycin-resistance gene present in pG$^+$Host9.

When FH CY-1/pAK149 is incubated at 38.0°C and plated on M17 Ery plates, surviving colonies will be strains in which pAK149 has integrated into the chromosome by homologous recombination between the *pyrF*$_{amber}$ fragment on pAK149 and the *pyrF* gene on the chromosome. The resulting strains have two copies of *pyrF*, one normal and one mutant. One such strain was purified and saved as FH CY-1/pAK149 Nr 1.

Bacteria cannot survive if they have two active replicons in their chromosome, so incubation of a strain like FH CY-1/pAK149 Nr 1 at 30°C will select for strains in which the integrated pG$^+$Host derivative has been removed. This can most easily occur by a second homologous recombination event. Depending on where this event occurs, the chromosome will either contain the normal *pyrF* gene or the *pyrF*$_{amber}$ gene. Strains in which the desired recombination event has occurred are found by screening survivors isolated on M17 plates at 30°C for their pyrimidine requirement. Strains that do not require pyrimidines for growth have the normal *pyrF* gene while strains that require pyrimidines have the *pyrF*$_{amber}$ gene. This process of integration and excision of pAK149 is illustrated in Figure 7. The overall result is that the *pyrF*$_{amber}$ gene originally on the plasmid and indicated by the dark line replaces the *pyrF* gene originally on the chromosome.

Pyr$^-$ survivors were tested for the presence of the amber mutation by amplifying the *pyrF* gene via PCR and confirming that the *Avr*II restriction site was still present. The DNA sequence of the *pyrF* gene was also determined following PCR amplification. One strain with exactly the desired DNA sequence of the *pyrF*$_{amber}$ gene was saved and deposited as CHCC4146 (DSM 12109). Thus, the strain CHCC4171 (DSM 12090) may be cured for the

vector pFG100 to obtain strain CHCC4146 as described in Dickely et al. 1995 and WO 95/10621.

4.7 Introduction of pFG100 and pFG200 into CHCC4146 and characterisation of the resulting strains, CHCC4171 and CHCC4223

Competent cells were made and electroporation with pFG100 were done. Ten colonies growing on minimal medium were purified and plasmid analysis showed that five of the colonies contained pFG100. The other five were spontaneous pyr^+ revertants. One strain was saved and designated CHCC4171 (DSM 12090).

Plasmid stability was tested and 100 of 100 colonies tested at each of 7, 14, 21, 28 and 35 generations in milk were found to contain pFG100. Thus, this plasmid is very stable in the FH CY-1 background. This is a significant improvement over the pFG1 system (WO 95/10621) which had over 90% plasmid-free cells after 40 generations in milk.

The acidification of milk by FH CY-1 and CHCC4171 was assessed as described above. The two strains were virtually indistinguishable and showed the high rate of acidification characteristic for FH CY-1 (Fig. 6). Furthermore, the acidification rate of strain CHCC4223 was as high as that of strain CHCC4171 and virtually indistinguishable from strain FH CY-1. This too was a significant improvement over the pFG1 system where the acidification rate for various FH CY-1 derivatives was considerably reduced.

4.8 Application of the food-grade cloning system

The functionality of the pFG200 was assessed by cloning of the *pepN* gene of *L. lactis* strain Wg2, encoding lysine aminopeptidase into the vector. The resulting plasmid, pFG202, was transformed into FA6-3 and CHCC4146 which then were grown in parallel with the host strains without plasmids (chromosomal *pepN* gene alone). After sonification of the harvested cells, the cell-free extracts were used to determine the PepN activity. The determined activities of lysine aminopeptidase are presented in Table 4 and show significant increases of PepN activity in both the MG1363 background strain (FA 6-3) and in the FHCY-1 background strain (CHCC4223).

Table 4. The effect of cloning the *pepN* gene into pFG200 by presenting the result of *pepN* overexpression in the MG1363 background strain (FA 6-3) and in the FHCY-1 background strain (CHCC4223).

Strain	**Activity of lysin aminopeptidase** [(a)]
FA 6-3	55
FA 6-3/pFG202	339
CHCC4146	17
CHCC4146/pFG202	211

[(a)] Determined as unit of lysine aminopeptidase per mg of protein

In addition, the lysin gene of the bacteriophage ML3 into pFG200 has been cloned. As expected, transformation of the generated plasmid construct into a host strain resulted in a much faster rate of lysis (no data shown).

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 15, line 19

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet [X]

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1B
D-38124 Braunschweig
Germany

Date of deposit	Accession Number
6 April 1998	DSM 12086

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet [ ]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
[ ] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12bis)

Additional sheet

In addition to the microorganism indicated on page 33 of the description, the following microorganisms have been deposited with

DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH
Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession numbers as stated below:

Accession number	Date of deposit	Description Page No.	Description Line No.
DSM 12087	6 April 1998	24	23
DSM 12088	6 April 1998	19	20
DSM 12089	6 April 1998	11	1
DSM 12090	6 April 1998	20	18
DSM 12091	6 April 1998	18	33
DSM 12108	16 April 1998	23	20
DSM 12109	17 April 1998	15	22
DSM 9190	6 May 1994	21	4

For all of the above-identified deposited microorganisms, the following additional indications apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms stated above only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

SEQUENCE LISTING

<110> Kim Ib Soerensen
Rasmus Larsen
Eric Johansen

<120> Novel food-grade cloning vectors and
their use in lactic acid bacteria

<130> 21030PC1

<150> DKA 0551/98
<151> 1998-04-21

<160> 27

<170> FastSEQ for Windows Version 3.0

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<400> 1
ggagccatgg cagagtggta atgcaacgga ctctaaatcc gtcgaaccgt gtaaagcggc 60
gcaggggttc aaatcccctt gactcctta 89

<210> 2
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template

<400> 2
cgaattcata tttgattaat gagaatatgg aacc 34

<210> 3
<211> 27
<212> DNA
<213> Artifical Sequence

<220>
<223> PCR primer amber-4 used was for the regeneration
of a PCR fragment with the plasmid pAK89 as a
template

<400> 3
cgggatcctt tcaggaaggt aattaac 27

<210> 4
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer amber-2 was used for the regeneration of a PCR fragment with the plasmid pAK89 as a template

<400> 4
cgaattcaac atttttgtat aaatatgcg 29

<210> 5
<211> 28
<212> DNA
<213> Atificial Sequence

<220>
<223> PCR primer amber-6 was used for the regeneration of a PCR fragment with the plasmid pAK89 as a template

<400> 5
gggaattcag gaaggtaatt aactatgg 28

<210> 6
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrF1 is based on the sequence of the pyrF gene of MG1363 and used to clone or sequence the pyrF gene of FH CY-1

<400> 6
gcagatctaa gcttgattca agaagtaaaa gaaggc 36

<210> 7
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrF2 is based on the sequence of the pyrF gene of MG1363 and used to clone or sequence the pyrF gene of FH CY-1

<400> 7
atagatctac tcgatgccaa gaatggaccg c 31

<210> 8
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrF3 is based on an amino acid sequence comparison of the pyrF gene product of a number of microorganisms and used to amplify a 550bp internal pyrF fragment

<400> 8
aaaggcctgt natngcnctn gayttycc 28

<210> 9
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrF4 is based on an amino acid sequence comparison of the pyrF gene product of a number of microorganisms and used to amplify a 550bp internal pyrF fragment

<400> 9
tggacgaatt ccnggngt 18

<210> 10
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrF5 is based on a 550bp internal pyrF fragment and used for inverse PCR with Sau3AI digested DNA

<400> 10
catagtaaac gacttgggg 19

<210> 11
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrF6 is based on a 550bp internal pyrF fragment and used for inverse PCR with Sau3AI digested DNA

<400> 11
tacgcacaaa aaaccgct 18

<210> 12
<211> 19
<212> DNA
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<220>
<223> Primer pyrF7 was uded for the sequencing of the amino terminal end of the pyrF gene

<400> 12
ggtcgccttt acttgcacc 19

<210> 13
<211> 20
<212> DNA
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<220>
<223> Primer pyrF8 was uded for the sequencing of the amino terminal end of the pyrF gene

<400> 13
gattatattg ttgtcggccg 20

<210> 14
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<220>
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<210> 15
<211> 25
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<220>
<223> The degenerate primer llagidB2 was uded for the sequencing of the carboxy terminal end of the pyrF gene

<400> 15
ggtngartgg aaygaraara thaay 25

<210> 16
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> PCR primer Fam5 was used to find a serine codon in the pyrF gene

<400> 16
cctcaaccta ggagaaaatt atgc 24

<210> 17
<211> 24
<212> DNA
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<220>
<223> PCR primer Fam6 was used to find a serine codon in the pyrF gene

<400> 17
tctcctaggt tgaggttaat tgtg 24

<210> 18
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Primer pyrD/BgIII was used for cloning the pyrFgene comprising an amber mutation

<400> 18
atagatctgc ttagaaaact tg 22

<210> 19
<211> 25
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<213> Artificial Sequence

<220>
<223> Primer pyrF11/BgIII was used for cloning the pyrFgene comprising an amber mutation

<400> 19
atagatctgc atgtaagcaa aaacc 25

<210> 20
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<213> Lactococcus lactis

<400> 20
Thr Gln Leu Thr Ser Thr Ser Glu Lys Ile Met Gln
1 5 10

<210> 21
<211> 36
<212> DNA
<213> Lactococcus lactis

<400> 21
acacaattaa cctcaacttc tgagaaaatt atgcaa 36

<210> 22
<211> 36
<212> DNA
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<400> 22
acacaattaa cctcaaccta ggagaaaatt atgcaa 36

<210> 23
<211> 937
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<400> 23
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gaccaaagcg agcattttat ccatagctaa aagaattgtc agcggagctg ataattctct 120
cgttcgttag cgaccaaagc gagcatttta tggatagcta aaagaattgt catcaaagct 180
gataattctg tcattaaata tttagaaaaa ggaagtagaa aaaatgcaag aaaatagacc 240
tgtcattgcc cttgatttcc ctgaattctc agacgtaaaa gattttctcg aaaaatttga 300
cccgtcagaa caattgtata ttaaactagg aatggaactt ttttacacgg ctgggcccca 360
agtcgtttac tatgtaaaat cgctcggcca cagtgtattc cttgatttaa aactccatga 420
tattccaaac accgttgaat cctcaatgcg tgttttagca cgtttgggat tggatatggt 480
taatgttcac gccgctggtg gtgttgaaat gatggttgca gctaaacgcg gtttagaggc 540
tggaacgcca gttggacggc aaaggccaaa attaattgcg gtcacacaat taacctcaac 600
ttctgagaaa attatgcaaa atgaccaaaa aattatgact agtcttgaag aatcggttat 660
taattacgca caaaaaaccg ctcaagcagg acttgacggt gtcgtttgtt cggcacatga 720
agttgaaaaa attaaagcag cgacatcgaa agaattcatt tgtctcacac caggaattcg 780
cccagaaggt gcaagtaaag gcgaccaaaa acgagtaatg acacctaaag aagcaagaac 840
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tagctatcat gcgataaaag cagaatggaa tcaataa 937

<210> 24
<211> 237
<212> PRT
<213> PyrF gene of Lactococcus lactis strain FH CY-1

<400> 24
Met Gln Glu Asn Arg Pro Val Ile Ala Leu Asp Phe Pro Glu Phe Ser
1 5 10 15
Asp Val Lys Asp Phe Leu Glu Lys Phe Asp Pro Ser Glu Gln Leu Tyr
20 25 30
Ile Lys Leu Gly Met Glu Leu Phe Tyr Thr Ala Gly Pro Gln Val Val
35 40 45
Tyr Tyr Val Lys Ser Leu Gly His Ser Val Phe Leu Asp Leu Lys Leu
50 55 60
His Asp Ile Pro Asn Thr Val Glu Ser Ser Met Arg Val Leu Ala Arg
65 70 75 80
Leu Gly Leu Asp Met Val Asn Val His Ala Ala Gly Gly Val Glu Met
85 90 95
Met Val Ala Ala Lys Arg Gly Leu Glu Ala Gly Thr Pro Val Gly Arg
100 105 110
Gln Arg Pro Lys Leu Ile Ala Val Thr Gln Leu Thr Ser Thr Ser Glu
115 120 125
Lys Ile Met Gln Asn Asp Gln Lys Ile Met Thr Ser Leu Glu Glu Ser
130 135 140
Val Ile Asn Tyr Ala Gln Lys Thr Ala Gln Ala Gly Leu Asp Gly Val
145 150 155 160
Val Cys Ser Ala His Glu Val Glu Lys Ile Lys Ala Ala Thr Ser Lys
165 170 175
Glu Phe Ile Cys Leu Thr Pro Gly Ile Arg Pro Glu Gly Ala Ser Lys
180 185 190
Gly Asp Gln Lys Arg Val Met Thr Pro Lys Glu Ala Arg Thr Ile Gly
195 200 205
Ser Asp Tyr Ile Val Val Gly Arg Pro Ile Thr Gln Ala Lys Asp Pro
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Val Ala Ser Tyr His Ala Ile Lys Ala Glu Trp Asn Gln
225 230 235

<210> 25
<211> 54
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<400> 25
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<210> 26
<211> 46
<212> DNA
<213> Artificial Sequence

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<223> Multiple cloning site in pFG100

<400> 26
ggatccacta gttctagagc ggccgccacc gcggtggagc tccagc 46

<210> 27
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<220>
<223> Multiple cloning site in pFG200

<400> 27
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cgggaattc 69

21030PC1

Chr. Hansen A/S
International Patent Application No. PCT/DK99/00209
Publication No. WO 99/54488

NEW CLAIMS, June 2000

1. A recombinant vector consisting essentially of lactic acid bacterial DNA, the vector comprising a gene coding for an amber suppressor which is a tRNA comprising the CUA anticodon and a replicon making the vector capable of replicating in a lactic acid bacterium, the vector having at least one of the following characteristics:

(i) when it is present in *Lactococcus lactis* strain FA4-1-1 (DSM 12086) having an amber mutation in the *pyrF* gene that is suppressible by the suppressor, it permits said strain to grow at 30°C at a doubling time of at the most 100 minutes in a minimal medium not containing pyrimidine sources;

(ii) when it is present in a strain of *Lactococcus lactis* FH CY-1 that has an amber mutation in the *pyrF* gene (strain CHCC4146, DSM 12109), the amber mutation being suppressible by the suppressor, it permits the strain to acidify milk under identical conditions at essentially the same rate of that of the parent strain (FH CY-1, DSM 12087);

(iii) it permits the *Lactococcus lactis* FA4-1-1 strain to grow at 30°C in a minimal medium not containing pyrimidine sources at a doubling time which is less than that for the *Lactococcus lactis* strain DN209 transformed with the vector pFG1.1 (DSM 12088), the pFG1.1 vector comprising a gene coding for a suppressor that is capable of suppressing the amber mutation in the DN209 strain, the transformed DN209 strain growing under conditions identical to those for the FA4-1-1 strain.

2. A vector according to claim 1 which has at least two of the characteristics (i) to (iii).

3. A vector according to claim 1 which has the characteristics (i) to (iii).

4. A vector according to claim 1 wherein the gene coding for the suppressor is derived from the chromosome of a lactic acid bacterium.

5. A vector according to claim 4 wherein the gene coding for a suppressor is under the control of a regulatable promoter.

6. A vector according to claim 5 wherein the regulatable promoter is a promoter not naturally related to the gene.

7. A vector according to claim 1 wherein the amber suppressor results from at least one change of nucleotide in an anticodon.

8. A vector according to claim 7 wherein the suppressor has two or three changes of nucleotide.

9. A vector according to claim 1 wherein the suppressor is a suppressor selected from the group consisting of a *supD*, *supE*, *supF*, *supP*, *supU* and a *supZ* suppressor.

10. A vector according to claim 1 wherein the replicon is derived from a *Lactococcus lactis* plasmid.

11. A vector according to claim 1 that comprises at least one unique restriction site.

12. A vector according to claim 1 that comprises a multiple cloning site.

13. A vector according to claim 1 which is a theta-replicating plasmid.

14. A vector according to claim 1 which is stably maintained for at least 35 generations in a lactic acid bacterium cultivated in a medium not containing pyrimidine sources.

15. A vector according to any of claims 1-14 which is selected from the group consisting of pFG100 deposited under the accession No. DSM 12091, a mutant, variant or derivative of pFG100, pFG200 deposited under the accession No. DSM 12108 and a mutant, variant or derivative of pFG200, said mutants, variants or derivatives essentially having the characteristics of the respective vector from which they are derived.

16. A vector according to claim 1 which comprises a gene coding for a desired gene product.

17. A vector according to claim 16 wherein the gene product is a peptidase selected from the group consisting of lysine-aminopeptidase, glutamyl-aminopeptidase, cysteine-aminopeptidase, iminopeptidase, X-prolyl-dipeptidyl aminopeptidase, endopeptidase, dipeptidase and tripeptidase.

18. A vector according to claim 16 wherein the gene product confers bacteriophage resistance to a lactic acid bacterial host cell.

19. A vector according to claim 16 wherein the gene product is a bacteriophage lysin.

20. A vector according to claim 19 wherein the gene coding for the bacteriophage lysin is derived from the bacteriophage ØvML3 as contained in DN209/pFG7 deposited under the accession No. DSM 12089.

21. A vector according to claim 16 wherein the gene product is involved in nisin synthesis or nisin resistance.

22. A lactic acid bacterium comprising a vector according to any of claims 1-21.

23. A lactic acid bacterium according to claim 22 that comprises an amber mutation being suppressible by the nonsense amber suppressor.

24. A lactic acid bacterium according to claim 23 wherein the amber mutation is located on a replicon different from the one containing the gene coding for the nonsense suppressor.

25. A lactic acid bacterium according to claim 22 wherein the suppressor is one suppressing a nonsense mutation which in the absence of a nonsense suppressor capable of suppressing the mutation, confers auxotrophy.

26. A lactic acid bacterium according to claim 25 wherein the nonsense mutation is in a gene involved in the synthesis of pyrimidine nucleotides.

27. A lactic acid bacterium according to claim 26 wherein the nonsense mutation is in a *pyr* gene.

28. A lactic acid bacterium according to claim 22 which is selected from the group consisting of a *Lactococcus* sp., *Streptococcus* sp., *Lactobacillus* sp., *Leuconostoc* sp., *Pediococcus* sp. and *Bifidobacterium* sp.

29. A lactic acid bacterium according to claim 28 which is *Lactococcus lactis*.

30. A lactic acid bacterium according to claim 29 which is *Lactococcus lactis* subsp. *lactis* strain FA4-1-1 containing pFG100, deposited under the accession No. DSM 12091 or *Lactococcus lactis* subsp. *lactis* strain CHCC4146 containing pFG200, deposited under the accession No. DSM 12108.

31. A lactic acid bacterium according to claim 22 wherein the vector is stably maintained for at least 35 generations when it is cultivated in a medium not containing pyrimidine sources.

32. An isolated pure culture of a lactic acid bacterium according to any of claims 22-31.

33. A composition comprising an isolated pure culture of a lactic acid bacterium as defined in claim 32, and a carrier.

34. A composition according to claim 33 containing at least 10^5 colony forming units of the lactic acid bacterium per g.

35. A method of using a composition as defined in claim 33 as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

36. A method of stably maintaining a vector according to claim 1 in lactic acid bacterial host cells growing in a particular environment, comprising providing said host cells as nonsense mutant cells having lost the capability of growing in said environment, and transformed with the vector according to claim 1 containing a nonsense suppressor gene encoding a gene product restoring the capability of the nonsense mutant cells to grow in said environment whereby, if the vector is lost from the lactic acid bacterial cells, the cells will not grow.

37. A method according to claim 36 wherein the nonsense mutant cells having lost the capability to grow are auxothrophic cells.

38. A method according to claim 37 wherein the nonsense mutant cells have a mutation in a gene involved in the synthesis of nucleotides.

39. A method according to claim 38 wherein the lactic acid bacterial host cells are *pyr* mutants.

40. A method according to claim 36 wherein the environment is a material selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

supD DNA fragment
BamHI
EcoRI
EcoRI
BamHI
ligation
pIL2608
(6690 bp)
tetR
rep
pFG100
3.2 kbp
supD
rep
Kpnl
Acc65I
ApaI
XhoI
SalI
HindIII
EcoRI
BamHI
SpeI
XbaI
EagI
NotI
SacII
BstXI
BpmI
SacI
Ecl136II
GGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTC
GGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGC

Fig. 1

2/10

Growth of strains harboring pFG1.1 and pFG100

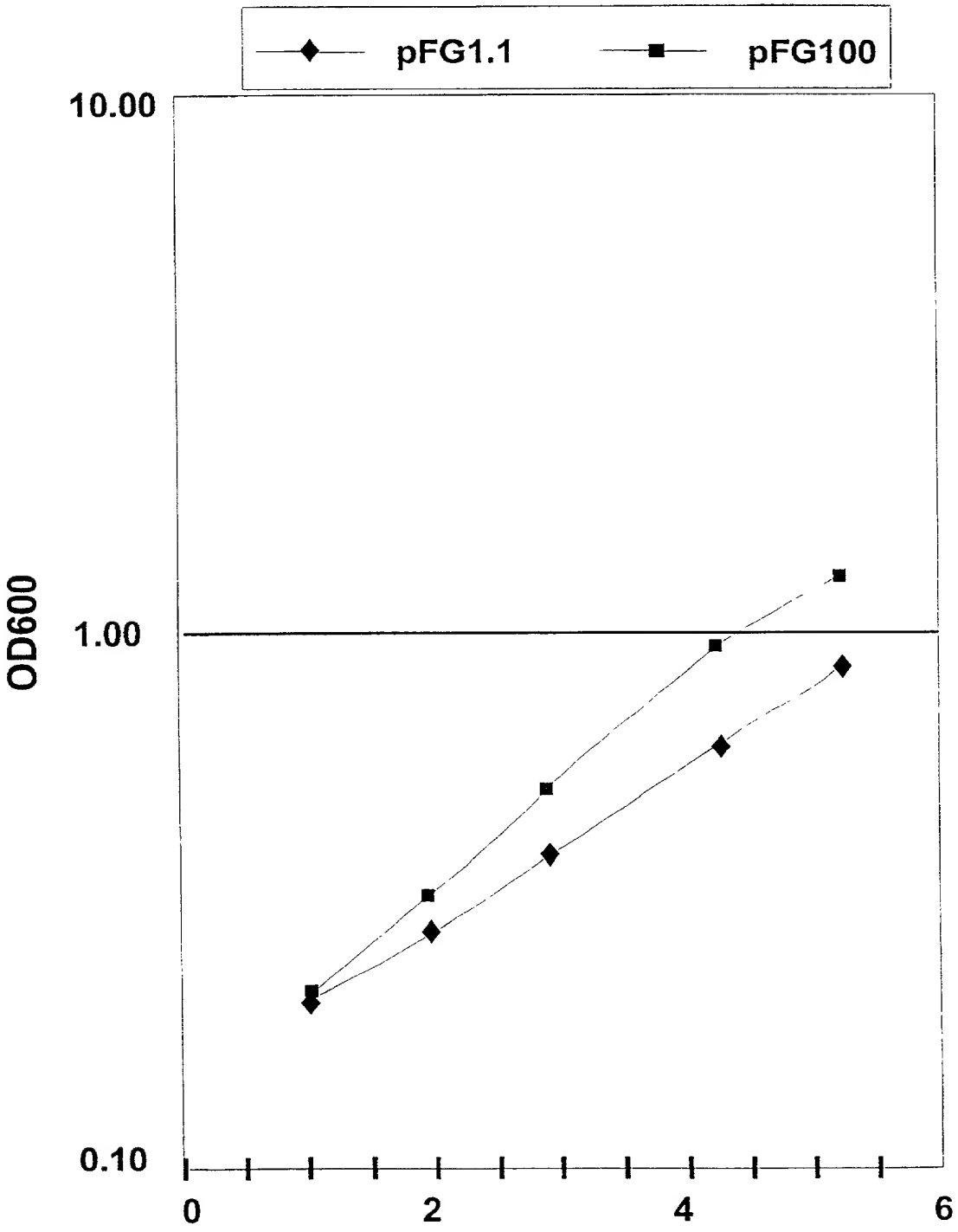


Fig. 2

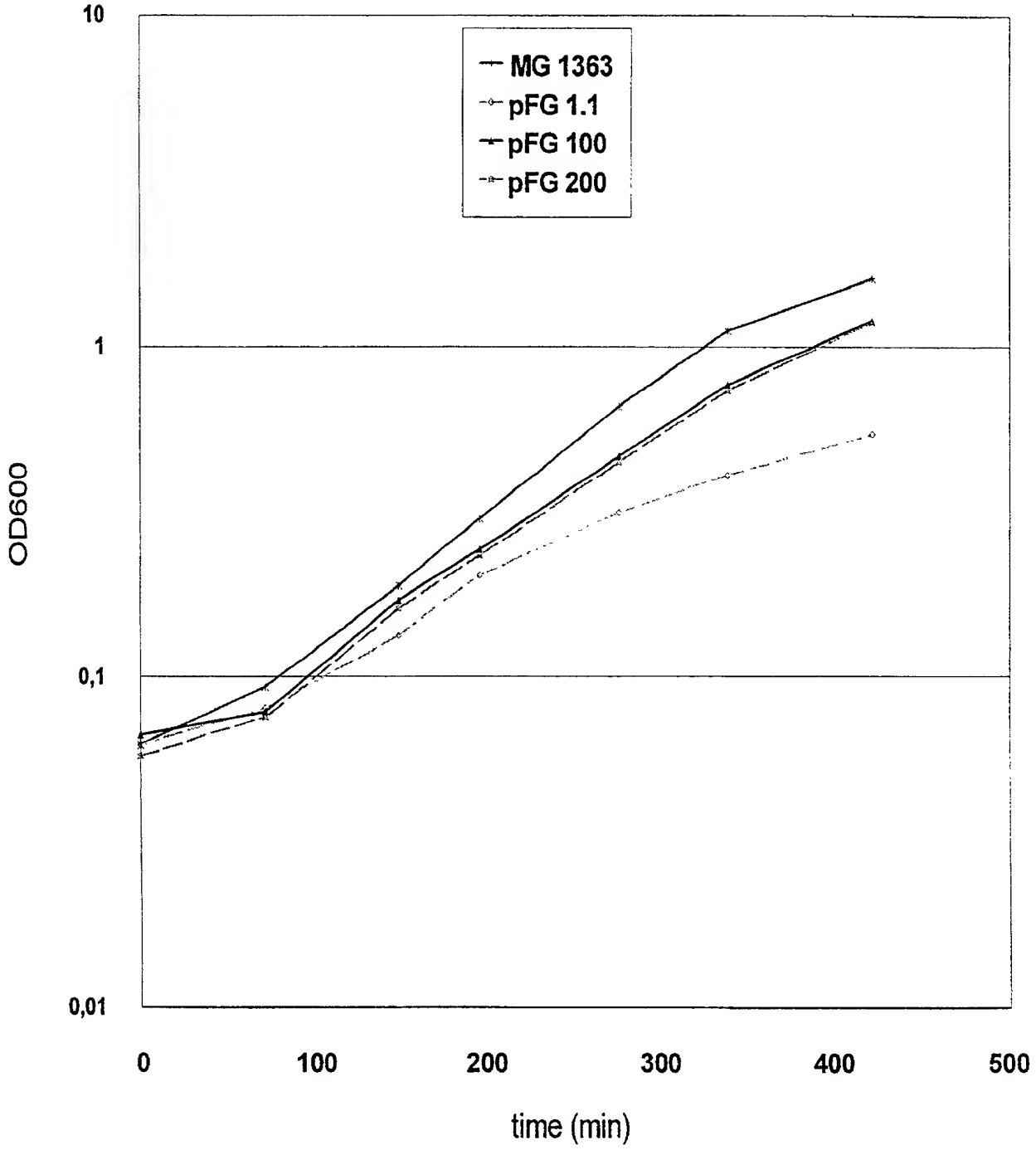

10
1
0,1
0,01
OD600
MG 1363
pFG 1.1
pFG 100
pFG 200
0
100
200
300
400
500
time (min)

Fig. 3

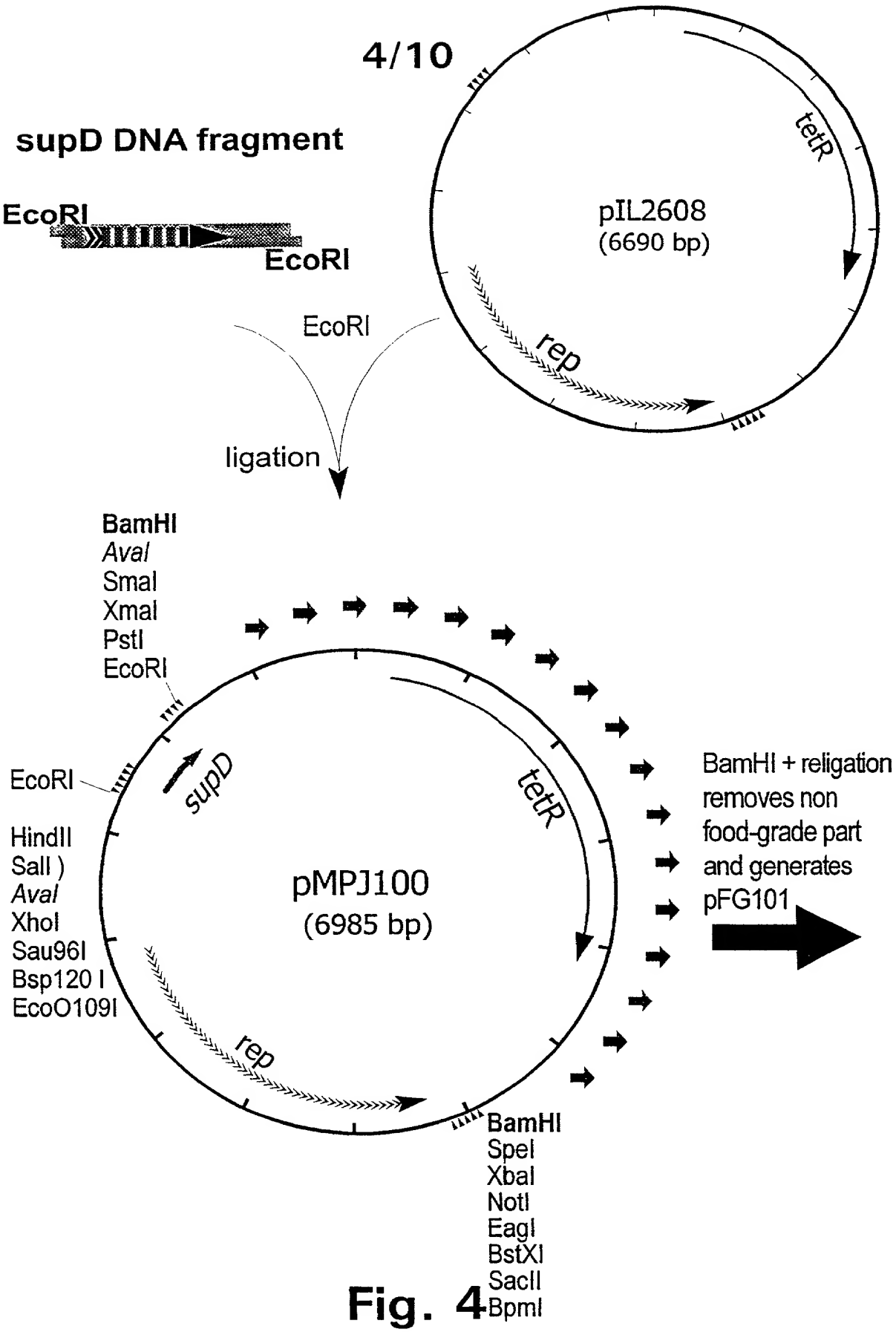
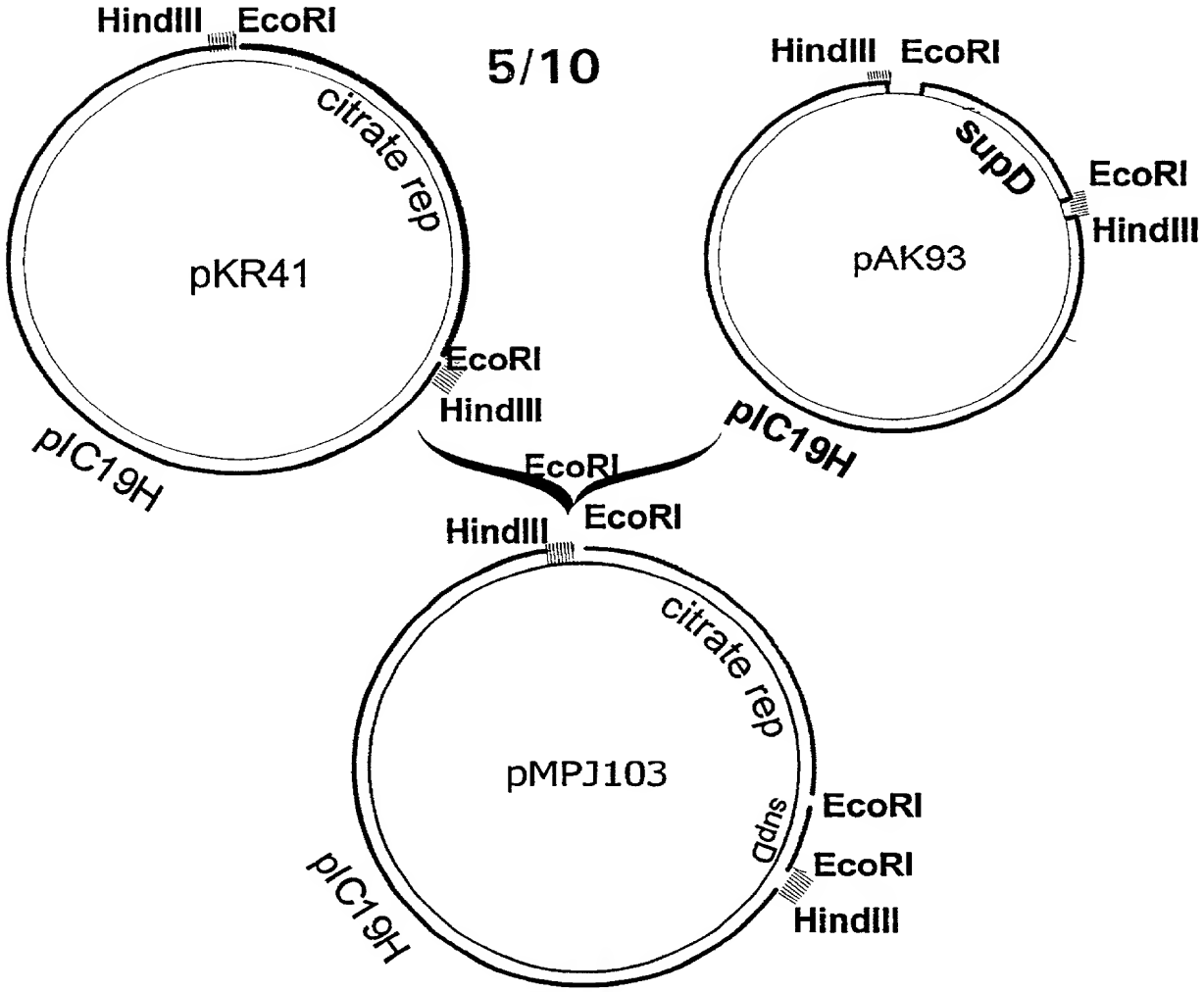

supD DNA fragment
EcoRI
EcoRI
pIL2608
(6690 bp)
tetR
rep
EcoRI
ligation
BamHI
AvaI
SmaI
XmaI
PstI
EcoRI
EcoRI
HindII
SalI)
AvaI
XhoI
Sau96I
Bsp120 I
EcoO109I
supD
tetR
pMPJ100
(6985 bp)
rep
BamHI
SpeI
XbaI
NotI
EagI
BstXI
SacII
BpmI
BamHI + religation
removes non
food-grade part
and generates
pFG101

Fig. 4



HindIII and ligation

XmaI
EcoRV BglII SacI SmaI
EcoRI XbaI XhoI NruI HindIII PstI SalI BamHI *EcoRI*
GAATTCATCGATATCTAGATCTCGAGCTCGCGAAAGCTTGGCTGCAGGTCGACGGATCCCCGGGAATTC

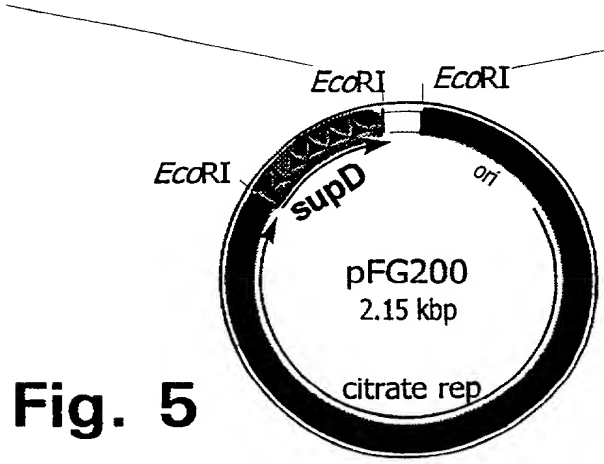


Fig. 5

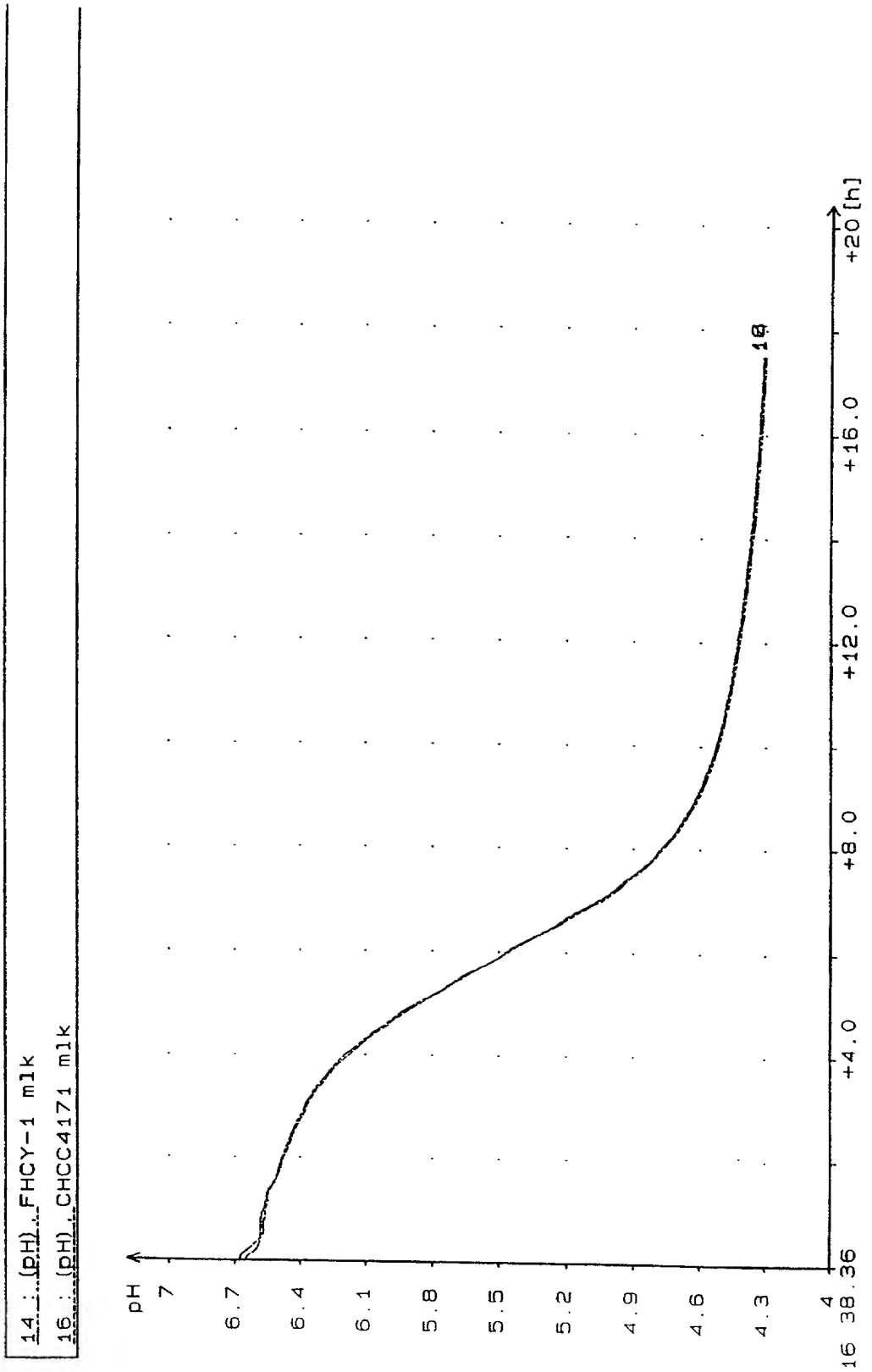

14 : (pH) FHCY-1 m1k
16 : (pH) CHCC4171 m1k
pH
7
6.7
6.4
6.1
5.8
5.5
5.2
4.9
4.6
4.3
4
16 38 36
+4.0
+8.0
+12.0
+16.0
+20 [h]
16

Fig. 6

	Color	LineStyle	Label
010			CHCC4223
011			CHCC4223.
012			CHCC4223..
013			FHCY-1
014			FHCY-1.

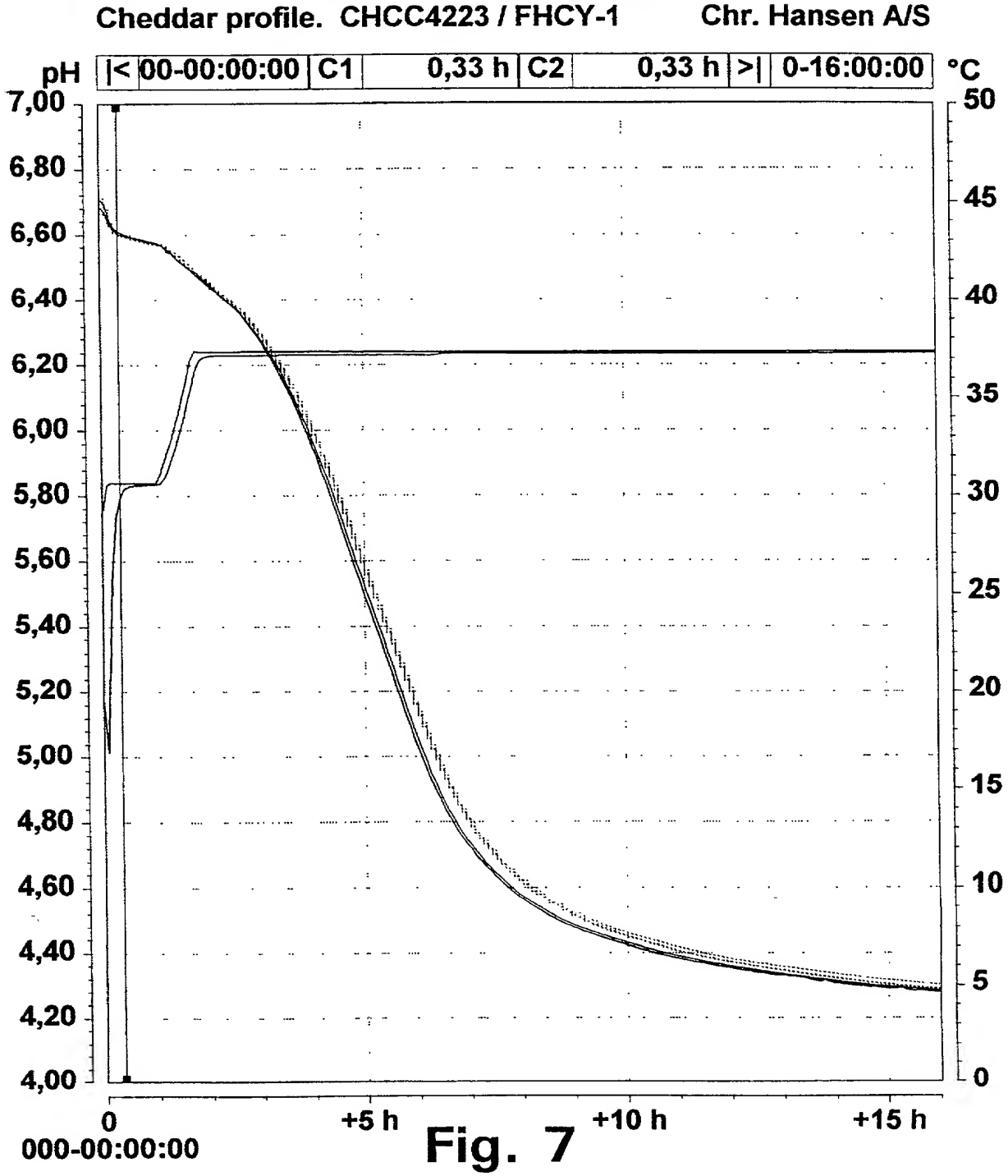


Fig. 7

```
   1 TGATTTTATTATTAGCTAAAATTACTGACAGCCTGTTTAATCATTCTGTCAGTAAAATGC  60
  61 GACCAAAGCGAGCATTTTATCCATAGCTAAAAGAATTGTCAGCGGAGCTGATAATTCTCT 120
 121 CGTTCGTTAGCGACCAAAGCGAGCATTTTATGGATAGCTAAAAGAATTGTCATCAAAGCT 160
 181 GATAATTCTGTCATTAAATATTTAGAAAAAGGAAGTAGAAAAAATGCAAGAAAATAGACC 240
                                                 M  Q  E  N  R  P  -

 241 TGTCATTGCCCTTGATTTCCCTGAATTCTCAGACGTAAAAGATTTTCTCGAAAAATTTGA 300
       V  I  A  L  D  F  P  E  F  S  D  V  K  D  F  L  E  K  F  D  -

 301 CCCGTCAGAACAATTGTATATTAAACTAGGAATGGAACTTTTTTACACGGCTGGGCCCCA 360
       P  S  E  Q  L  Y  I  K  L  G  M  E  L  F  Y  T  A  G  P  Q  -

 361 AGTCGTTTACTATGTAAAATCGCTCGGCCACAGTGTATTCCTTGATTTAAAACTCCATGA 420
       V  V  Y  Y  V  K  S  L  G  M  S  V  F  L  D  L  K  L  M  D  -

 421 TATTCCAAACACCGTTGAATCCTCAATGCGTGTTTTAGCACGTTTGGGATTGGATATGGT 480
       I  P  N  T  V  E  S  S  M  R  V  L  A  R  L  G  L  D  M  V  -

 481 TAATGTTCACGCCGCTGGTGGTGTTGAAATGATGGTTGCAGCTAAACGCGGTTTAGAGGC 540
       N  V  H  A  A  G  G  V  E  M  M  V  A  A  K  R  G  L  E  A  -

 541 TGGAACGCCAGTTGGACGGCAAAGGCCAAAATTAATTGCGGTCACACAATTAACCTCAAC 600
       G  T  P  V  G  R  Q  R  P  K  L  I  A  V  T  Q  L  T  S  T  -

 601 TTCTGAGAAAATTATGCAAAATGACCAAAAAATTATGACTAGTCTTGAAGAATCGGTTAT 660
       S  E  K  I  M  Q  N  D  Q  K  I  M  T  S  L  E  E  S  V  I  -

 661 TAATTACGCACAAAAAACCGCTCAAGCAGGACTTGACGGTGTCGTTTGTTCGGCACATGA 720
       N  Y  A  Q  K  T  A  Q  A  G  L  D  G  V  V  C  S  A  M  E  -

 721 AGTTGAAAAAATTAAAGCAGCGACATCGAAAGAATTCATTTGTCTCACACCAGGAATTCG 780
       V  E  K  I  K  A  A  T  S  K  E  F  I  C  L  T  P  G  I  R  -

 781 CCCAGAAGGTGCAAGTAAAGGCGACCAAAAACGAGTAATGACACCTAAAGAAGCAAGAAC 840
       P  E  G  A  S  K  G  D  Q  K  R  V  M  T  P  K  E  A  R  T  -

 841 AATTGGTTCAGATTATATTGTTGTCGGCCGTCCAATTACCCAAGCAAAAGATCCAGTAGC 900
       I  G  S  D  Y  I  V  V  G  R  P  I  T  Q  A  K  D  P  V  A  -

 901 TAGCTATCATGCGATAAAAGCAGAATGGAATCAATAA 937
       S  Y  H  A  I  K  A  E  W  N  Q  *
```

Fig. 8

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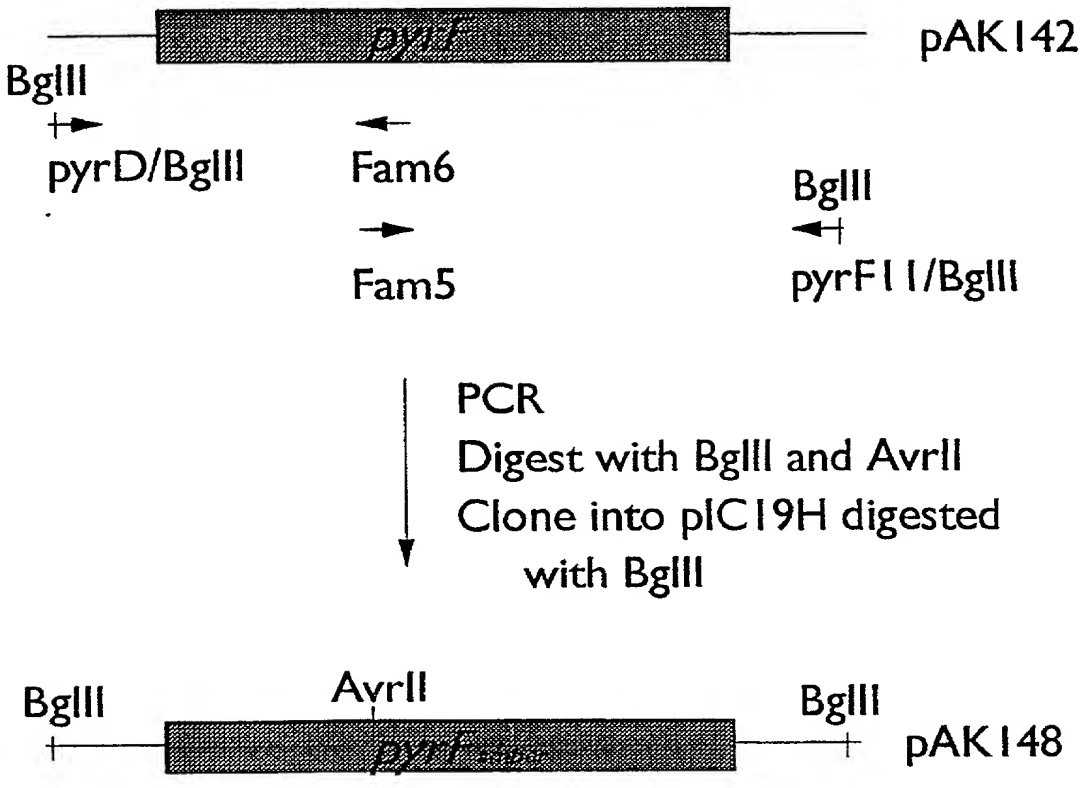
pyrF
pAK142
BglII
pyrD/BglII
Fam6
Fam5
BglII
pyrF11/BglII
PCR
Digest with BglII and AvrII
Clone into pIC19H digested
with BglII
BglII
AvrII
BglII
pyrF
pAK148

Fig. 9

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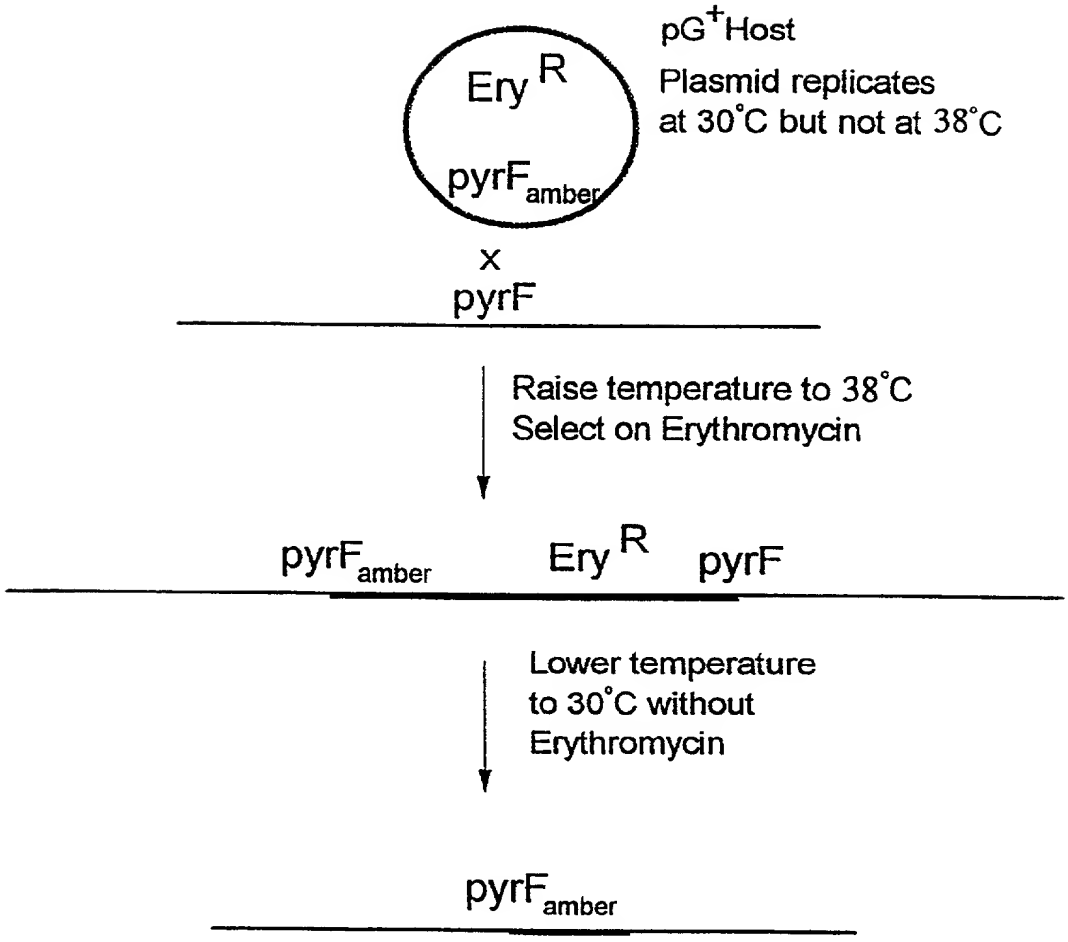
pG+Host
Ery R
pyrFamber
Plasmid replicates
at 30°C but not at 38°C
x
pyrF
Raise temperature to 38°C
Select on Erythromycin
pyrFamber
Ery R
pyrF
Lower temperature
to 30°C without
Erythromycin
pyrFamber

Fig. 10

21030 DC 2

Atty. Dkt. No. 030307/0191

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

FOOD-GRADE CLONING VECTOR AND THEIR USE IN LACTIC ACID BACTERIA

(Attorney Docket No. 030307/0191)

the specification of which (check one)

___ is attached hereto.

__X__ was filed on April 21, 1998 as United States Application Number or PCT International Application Number PCT/DK99/00209 and was amended on _____ (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date
60/082,555	April 21, 1998

I HEREBY CLAIM the benefit under Title 35, United States Code, §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

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to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith.

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1 - 00

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2 - 00

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3 - 00

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